Eesti Maaülikool
Estonian University of Life Sciences

ASSOCIATIONS BETWEEN METABOLIC PROFILE AND COAGULATION ABILITY OF BOVINE MILK, EFFECT OF FEEDING AND LACTATION STAGE

LEHMAPIIMA METABOOLSE PROFIILI JA LAAPUMISE VAHELISED SEOSED, SÖÖTMISE JA LAKTATSIOONIPERIOODI MÕJU

HEDI HARZIA

A Thesis
for applying for the degree of Doctor of Philosophy in Animal Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks loomakasvatuse erialal

Tartu 2013

EESTI MAAÜLIKOOL
ESTONIAN UNIVERSITY OF LIFE SCIENCES

Eesti Maaülikool
Estonian University of Life Sciences

ASSOCIATIONS BETWEEN METABOLIC PROFILE AND COAGULATION ABILITY OF BOVINE MILK, EFFECT OF FEEDING AND LACTATION STAGE

LEHMAPIIMA METABOOLSE PROFIILI JA LAAPUMISE VAHELISED SEOSED, SÖÖTMISE JA LAKTATSIOONIPERIOODI MÕJU

HEDI HARZIA

A Thesis
for applying for the degree of Doctor of Philosophy in Animal Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks loomakasvatuse erialal

Tartu 2013

Institute of Veterinary Medicine and Animal Sciences,
Department of Nutrition and Animal Products Quality,
Estonian University of Life Sciences

According to verdict No 158 of October 28, 2013, the Doctoral Committee of the Agricultural and Natural Sciences of the Estonian University of Life Sciences has accepted the thesis for the defence of the degree of Doctor of Philosophy in Animal Science.

Opponent: Rossella Di Palo, DVM, PhD, Prof.
University of Naples Federico II, Italy

Supervisors: Olav Kärt, Dr Sci, Prof. *Emer.*
Estonian University of Life Sciences

Ursel Soomets, PhD, Prof.
University of Tartu

Kalle Kilk, PhD, Senior Research Fellow
University of Tartu

Defence of the thesis:
Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, room 1A5, Kreutzwaldi 5, Tartu on December 20, 2013, at 10 o'clock.

The English language was edited by David Arney and the Estonian by Eve-Liis Abroi.

Publication of the thesis has been granted by Estonian University of Life Sciences and by the Graduate School in Biomedicine and Biotechnology (1.2.0401.09-0073).

ISBN 978-9949-536-07-8 (trükis)
ISBN 978-9949-536-08-5 (pdf)

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is a summary of the following four original publications (I-IV). The papers are referred to in the text by Roman numerals.

I **Harzia, H.,** Kilk, K., Jõudu, I., Henno, M., Kärt, O., Soomets, U. 2012. Comparison of the metabolic profiles of noncoagulating and coagulating bovine milk. Journal of Dairy Science, 95(2), 533 – 544.

II Ilves, A., **Harzia, H.,** Ling, K., Ots, M., Soomets, U., Kilk, K. 2012. Alterations in milk and blood metabolomes during first months of lactation in dairy cows. Journal of Dairy Science, 95(10), 5788 - 5797.

III **Harzia**, **H.,** Kilk, K., Ariko, T., Kass, M., Soomets, U., Jõudu, I., Kaart, T., Arney, D., Kärt, O., Ots, M. 2013. Crude glycerol as glycogenic precursor in feed; effects on milk coagulation properties and metabolic profiles of dairy cows. Journal of Dairy Research, 80 (2), 190-196.

IV **Harzia, H.**, Ilves, A., Ots, M., Henno, M., Jõudu, I., Kaart, T., Ling, K., Kärt, O., Kilk, K., Soomets, U. 2013. Alterations in milk metabolome and coagulation ability during the lactation of dairy cows. Journal of Dairy Science, 96 (10), 6440-6448.

Authors' contributions to the papers as follows:

Publication	I	II	III	IV
Idea and planning of experiment	**HH**, MH	AI, KL	**HH**, MK, MO, OK, TA	**HH**, MH
Analyses of milk samples	**HH**, KK	AI, **HH**, KK	**HH**	**HH**, AI
Data analyses and interpretation	**HH**, KK	AI, KK, TK	**HH**, KK, TK	**HH**, AI, TK
Manuscript preparation	All	All	All	All

AI – Aire Ilves; **HH - Hedi Harzia;** KK – Kalle Kilk; KL – Katri Ling; MH – Merike Henno; MK – Marko Kass; MO – Meelis Ots; OK – Olav Kärt; TA – Tiia Ariko; TK – Tanel Kaart; All – all authors of the paper.

ABBREVIATIONS

amu	atomic mass unit
CP	crude protein
DM	dry matter
E_{30}	curd firmness at 30 min after rennet addition
EARC	Estonian Animal Recording Centre
EHF	Estonian Holstein breed
EMS	enhanced mode
EN	Estonian Native breed
ER	Estonian Red breed
GLM	general linear mixed model
HILIC	hydrophilic interaction liquid chromatography
LC-MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
m/z	mass to charge ratio
ME	metabolisable energy
MJ	megajoule
MP	metabolisable protein
MRM	multiple reaction monitoring mode
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
PCA	principal component analysis
PLSC	partial least squares correlation
RCT	rennet coagulation time
SCC	somatic cell count
SCS	somatic cell score
T0	control ration
T1	1 kg of crude glycerol added to the ration
T2	2 kg of crude glycerol added to the ration
T3	3 kg of crude glycerol added to the ration
TCA	trichloroacetic acid
TMR	total mixed ration
VFA	volatile fatty acids

1. INTRODUCTION

The main dairy product produced in Europe is cheese. In 2011, 36% of the 140 million tons of milk produced was converted into cheese (Eurostat, 2012), and most of those cheeses were produced by enzymatic coagulation. The coagulation ability of milk influences cheese yield and quality, and is therefore of high economic interest (Bittante *et al.*, 2012). For the dairy industry it is important that the milk forms a firm curd and coagulates evenly in the most desirable time preventing losses of fines (Cecchinato *et al.*, 2013), because cheesemaking efficiency is rated by the amount of milk solids lost in whey. The time can be optimised by using a higher enzyme content, but, an undesirable outcome of this could be decrease in yield and an increased amount of bitter peptides in the end product (Cecchinato *et al.*, 2013). Coagulation ability is therefore a widely studied heritable trait (Ikonen *et al.*, 1999; Cassandro *et al.*, 2008; Vallas *et al.*, 2010; Bittante *et al.*, 2012), and is affected, in addition to genes, by other factors, including stage of lactation, breed, and feeding (Jõudu, 2008). To date there are still neither genetic, nor other factors which could explicitly explain, and allow the prediction of, the coagulation properties of milk. It is an emerging field of high interest to improve the efficiency of cheese production by improving raw milk rennet coagulation properties, and investigation of those factors affecting coagulation at the molecular level.

Globally, bovine milk metabolite research is mainly based on the need to sustain safe food for human consumption free of pesticides, mycotoxins and antibiotics and their metabolites (Gentili *et al.*, 2005; Sørensen, Elbæk, 2005; Blasco *et al.*, 2009). Historically, metabolomic analyses began with the use of nuclear magnetic resonance (NMR), known as a non–destructive technique, as well for high-throughput and minimal sample preparation (Dettmer *et al.*, 2007). Nowadays the main technique used to analyse metabolites, besides NMR, is mass spectrometry (MS), which is also a highly sensitive and selective method (Dettmer *et al.*, 2007).

In addition to xenobiotics, normal biochemical variability of milk has been investigated (Boudonck *et al.*, 2009; Klein *et al.*, 2010). Through analysis of milk composition and its metabolites, it is possible to evaluate animals' health and nutritional status, and thereby make more accurate decisions about their treatment or feeding. Although in clinical

diagnostics and toxicology blood or urine are most often used to assess body condition, the most interesting body fluid of the dairy cow is milk. This is due to the noninvasiveness of collection, milk samples are easier to gather than blood samples and in precision farming all analyses regarding animals' health can be made at milking. To date on-farm accessible disease prediction is still mainly based on observing subclinical or clinical signs. There are good indicators for the early diagnosis of ketosis by measuring milk β-hydroxybutyrate content, but other feeding-related metabolic diseases have no sufficient noninvasive methods of analysis (Bjerre-Harpoth *et al.*, 2012). Using NMR, the prognoses of ketoses have been associated with the glycerophosphocholine to phosphocholine ratio (Klein *et al.*, 2012). To date there are few studies describing the common milk metabolites as indicators of cows' milk technological properties (Sundekilde *et al.*, 2011; **paper I**).

This study represents the first large screen metabolomic profiling of bovine milk samples in Estonia. In the long run this method could be used to identify the most suitable milk for cheesemaking based on a few easily detectable biomarkers or, a set of these. If the results indicate that the set of detected metabolic markers are alterable through feeding, or useful in selection for milk, it would provide an economic advantage to the dairy industry, and dairy production by improving dairy cattle productivity.

2. REVIEW OF THE LITERATURE

2.1. Milk coagulation

The most important stage in cheesemaking is the coagulation of milk by a renneting enzyme (e.g. chymosin). Milk coagulation is a process divided into two stages where, during the first, κ-casein goes through enzymatic hydrolysis while chymosin cuts it into two parts – para-κ-casein and caseinomacropeptide. When the macropeptide is discarded into whey the second stage can be initiated and the aggregation of casein micelles takes place. These phases can overlap in milk depending on the conditions present. The aggregation of casein micelles is temperature sensitive, as it takes place at temperatures of 18 ºC and over. During gelation, casein micelles are rearranged into three-dimensional networks, which enable the dehydration of milk during the cheesemaking process, and produces the desired characteristics of the cheese (Lucey, 2002). The first stage can be measured in minutes, the time from the addition of rennet to milk until the beginning of coagulation, and is described as rennet coagulation time (RCT). The second stage is usually measured as curd firmness, in mm after 30 min of enzyme addition (E_{30}) (Lucey, 2002; Kübarsepp *et al.*, 2005a; **paper I**). There have been studies (Cipolat-Gotet *et al.*, 2012; Cecchinato *et al.*, 2013) where curd firmness was measured after 45 min of rennet addition. In addition to different times, there are also different apparatus used to determine the coagulation ability of milk, described below.

2.1.1. Assessing coagulation ability

Different techniques exist to measure the abovementioned curd firmness and other coagulation parameters. The use of these is quite country specific, as the Formograph is used in Italy and Finland (Ikonen *et al.*, 1999; Tyrisevä *et al.*, 2003; Bittante *et al.*, 2013), while the Optigraph (Kübarsepp *et al.*, 2005a; Vallas *et al.*, 2010; **paper I**) is used in Estonia. Studies comparing both methods have been made in Italy and Estonia separately (Kübarsepp *et al.*, 2005a; Cecchinato *et al.*, 2013) and also in cooperation (Pretto *et al.*, 2011). The rheometer is used to measure coagulation ability in Denmark (Frederiksen *et al.*, 2011; Sundekilde *et al.*, 2011).

Of the aforementioned techniques, the Formogarph (Foss Electric A/S, Hillerød, Denmark) is a mechanical device, where a pendulum is submerged into the sample and, the viscosity is measured by the oscillation of the pendulum. Results, expressed as RCT, k_{20} (the time required to form a curd) and curd firmness, are recorded and displayed graphically (Cipolat-Gotet *et al.*, 2012). The Optigraph (Alliance Instruments, Frepillon, France) measures the gel strength by a near infrared optical signal. The output graph expresses RCT and E_{30} data (Kübarsepp *et al.*, 2005a). Both devices can measure 10 samples at a time, and the data acquired can be compared using a formula devised by Kübarsepp *et al.* (2005a) or using special software (Cipolat-Gotet *et al.*, 2012). Rheological parameters such as RCT, storage modulus as the maximum coagulum strength (G'_{max}) and curd firming rate (CFR), describing viscosity and/or elastic properties of milk, are measured by a rheometer, and the data are also expressed visualized with an output graph (Frederiksen *et al.*, 2011). Unfortunately there are limits to the number (e.g. n = 1 or 4) of samples analysed. Nevertheless, a study conducted by Klandar *et al.* (2007) showed strong correlations between rheometer and near infrared spectra analyses.

2.1.2. Factors affecting coagulation ability

According to coagulation ability, milk can be divided into non- (E_{30} = 0 mm), poor ($0 < E_{30} < 20$ mm), and good ($20 \text{ mm} \leq E_{30}$) milk coagulation (Tyrisevä *et al.*, 2004). Non-coagulating milk is not capable of forming a curd within 30 minutes after rennet addition, and poor coagulating milk has a weak curd (Tyrisevä *et al.*, 2004). Firmer coagulum can be obtain by a shorter RCT, as there is more time left for the curd to firm further, and when the coagulum has less time to firm within 30 min, the curd will be weaker (Cassandro *et al.*, 2008). Good coagulation properties provide good conversion of milk solids to cheese and profits to the dairy companies, and therefore poor or non-coagulation milks are not preferred. Poor coagulation was a problem in Italy and in Finland, where about 9.7% of Italian Holstein-Friesian, and 8.6% of Finnish Ayrshire cows, produced non-coagulating milk on at least one occasion during lactation (Tyrisevä *et al.*, 2004; Cassandro *et al.*, 2008). A study by Frederiksen *et al.* (2011) found that 20% of analysed Danish milk samples had poor or non-coagulation. A review by Jõudu *et al.* (2009) also observed poor coagulating milk in Estonia, where about 8-9% of milk samples did not coagulate and 17-20% coagulated poorly; and provided

an overview of milk rennet coagulation properties among dairy cattle in Estonia, emphasising breeds and κ-casein genotypes (e.g. AA, AE, EE, BB). In addition to κ-casein genotypes and breed there are other factors affecting milk coagulation ability, described below.

This heritable (Ikonen *et al.*, 1999; Cassandro *et al.*, 2008; Vallas *et al.*, 2010; Bittante *et al.*, 2012) milk coagulating trait is associated with different genes, e.g. according to Tyrisevä *et al.* (2008) non-coagulation is caused by loci on chromosomes 2 (BMS1126) and 18 (BMS1355). Of the different protein genotypes, the non-favourable rennet coagulation parameters are affected by the κ-casein AA, AE, and EE genotypes, and the shorter RCT and firmer curd is associated with the BB genotype (Schaar, 1984; Jõudu *et al.*, 2009). Of environmental factors stage of lactation also has an affect; coagulation properties are best at the beginning, worst in the middle, and good at the end of lactation (Ostersen *et al.*, 1997; Summer *et al.*, 2003; Tyrisevä *et al.*, 2003). As mentioned previously Holstein cows (Cassandro *et al.*, 2008; Jõudu *et al.*, 2009) tend to produce more non-coagulating milk than other breeds, which can affect milk coagulation at the herd level. Herd management through animal health also has an affect on milk coagulation, as mastitic or milk with elevated SCC tends to coagulate poorly or not at all; higher SCC content alters milk protein and fatty acid proportion, and increases enzymatic activity (Politis *et al.*, 1989; Ogola *et al.*, 2007; Sharif, Muhammad, 2008). As the cows get older and lactation number increases, the curd gets weaker (Schaar, 1984; Tyrisevä *et al.*, 2003). It has been observed that coagulation ability can also be altered by feeding (**paper III**), and during the outdoor period the protein content of milk is higher (Grimley *et al.*, 2009), and therefore the curd is firmer (Jõudu *et al.*, 2009). Of the main milk characteristics, a decrease in the pH increases the RCT, but higher fat and lactose contents result in a shorter RCT, and firmer curd results from higher calcium and protein contents (Kübarsepp *et al.*, 2005b; Jõudu *et al.*, 2008). These factors have been widely studied, but none of them can unambiguously explain the non-coagulation of milk.

2.2. Metabolomics

Metabolomics is the identification, quantification and characterisation of small molecules (< 1,500 Da) found in an organism (Wishart, 2008). In addition to genomics, transciptomics and proteomics, it is one of

the "-omics" sciences trying to comprehend the system of a living organism at the molecular level. Globally, and especially in Europe, food metabolites research is based on legal obligations, which provide exact requirements for mycotoxins (Commission Regulation, 2006a), heavy metals, 3-monochloropropane-1,2-diol, inorganic tin, benzo(a)pyrene (Commission Regulation, 2007), dioxin, polychlorinated biphenyls (Commission Regulation, 2006b) and nitrate (Commission Regulation, 2006c) levels in food in order to guarantee high quality foods for human health. The EU has thereby taken measures to minimize contaminants in foodstuffs. Because of the many different low molecular weight substances in food, metabolomics has been applied to food science and nutrition.

2.2.1. Acquiring metabolic data

Metabolomics is a currently expanding field of research, which applies many different technologies, such as mass spectrometry (MS), nuclear magnetic resonance spectrometry (NMR), capillary electrophoresis and liquid or gas chromatography coupled with MS (Wishart, 2008). Unfortunately none of these tools can analyze all the metabolites single-handedly. A study by Dettmer *et al.* (2007) showed that the most common techniques used to analyse metabolites in various substances are NMR and MS. Unfortunately NMR requires metabolites in abundance, and complex matrices can make metabolite identification complicated (Dettmer *et al.*, 2007).

MS has high selectivity and sensitivity, therefore the metabolite of interest does not have to be so abundant (Dettmer *et al.*, 2007). It allows the metabolic profile to be obtained rapidly, and with minimal sample preparation, and is able to measure a large range of metabolites from a complex matrix such as milk. In combination with statistical methods it allows the variances between different groups to be determined (Boudonck *et al.*, 2009). It also allows the analysing of hundreds if not thousands of metabolites in milk, giving exact masses of molecules or fragments. In the case of very complex samples, or the presence of interfering compounds, the sample of interest may go through sample preparation (e.g. liquid-liquid or solid-phase extraction) and separation methods (e.g. capillary electrophoresis, gas chromatography or high-performance liquid chromatography) before MS detection (Dettmer *et al.*, 2007). Depending on the type of sample and MS apparatus the

sample is ionized by different methods for different compounds, e.g. matrix-assisted laser desorption is better for compounds > 600 Da, because of the interference by the matrix, electrospray ionization does not have this kind of limitation (Hop, Bakhtiar, 1997).

2.2.2. Mass spectrometry-based approaches

MS-based metabolomics uses two different approaches: (1) targeted profiling, and/or (2) fingerprinting (untargeted profiling, global analyses). Targeted profiling is used when the metabolites of interest are known before analysis, while fingerprinting establishes identification of the metabolite afterwards. According to Scalbert *et al.* (2009) both of these approaches have their advantages and disadvantages. Targeted profiling is an easy and fast method used when standardized methods or procedures are established, databases for identification are created or a stable isotope-labelled internal standard is used. Internal standards also have another use to make calibration curves in order to quantify the metabolites of interest. Fingerprinting usually has no fixed instructions, and identification of the metabolites of interest requires a good library. To make identification easier databases have been created, some commercialised as software and others as freeware, such as MassBank (http://www.massbank.jp/), the Human Metabolome Database (HMDB; http://www.hmdb.ca/) and the METLIN Metabolome Database (http://metlin.scripps.edu/).

2.3. Mass spectrometric analyses of milk

Milk metabolites, including naturally occurring metabolites and xenobiotics, have been widely studied. Phytoestrogenes, as a result of different farming practices and influence on feedstuffs, have been investigated by Antignac *et al.* (2004) and a good review of different analytical separation and detection methods for flavonoids has been presented by de Rijke *et al.* (2006). Veterinary drug residues in animal-food products have also been studied with different liquid chromatography mass spectrometry (LC-MS) methods (Gentili, 2007). The methods used to determine mycotoxins (Sorensen, Elbaek, 2005) and pesticides (Blasco *et al.*, 2009) are routinely used. The food industry and healthcare sectors have a big interest in biologically active ingredients in milk to enrich infant formulas. Milk oligosaccharides are the biologically active

ingredients of greatest interest. Therefore structural and nutritional aspects of milk oligosaccharides (Urashima *et al.*, 2001; Tao *et al.*, 2008; Tao *et al.*, 2009), as well as amino acids, lipids, nucleotides, vitamins, cofactors and short peptides have been investigated using MS (Boudonck *et al.*, 2009). Besides assuring high nutrient density, the contents of harmful chemicals in infant formulas and consumer milk have also been investigated (Mortensen *et al.*, 2005). Not only at the beginning, but also throughout lactation, the changes in the metabolic composition of milk have been investigated (Klein *et al.*, 2010; Klein *et al.*, 2012; **paper II**). Studies analysing the milk metabolome for milk technological properties are scarce, with the exception of Sundekilde *et al.* (2011) and **paper I**. Both studies observed the differences in metabolic profiles of milk with different coagulation abilities.

3. HYPOTHESIS AND AIMS OF THE STUDY

The coagulation ability of milk is of great interest, and as the most important property of cheese milk it has a high economical and gastronomical effect on the end product. It is a widely studied heritable trait, which can be affected by a range of different environmental factors as well as genes. Unfortunately data about the reasons for poor coagulation are still unreliable. Therefore the main objective globally is to determine the main causes affecting unfavourable coagulation ability. A novel approach is to study the low molecular weight compounds associated with coagulation ability, and thereby to better understand the processes occurring at the molecular level.

Hypothesis

1. Through understanding the molecular composition of milk, milk technological properties can be estimated.
2. The coagulation ability of milk and its relationship with the metabolic profile is associated with lactation stage and nutrition of the cow.

The aims of this study were:

1. To develop a fast and robust protocol for milk pre-treatment liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis for low molecular compounds (**I, II**), which would allow the pinpointing of specific metabolic markers describing coagulation ability, thus providing better understanding about the mechanisms responsible for milk coagulation at the molecular level.

2. To get an overview of the metabolic profile of milk with different coagulation abilities, and to determine the specific metabolic markers associated with coagulation ability, by analysing the milk samples with extremes of coagulation ability (non- and well-coagulating) (**I**).

3. To verify the effect of feeding on coagulation ability and the metabolic profile, by analysing milk samples from dairy cows fed with crude glycerol (**III**).

4. To determine the extent to which coagulation ability of milk and its relationship with the metabolic profile changes during lactation (**IV**).

4. MATERIAL AND METHODS

4.1. Data collection

4.1.1. Experimental design

The experimental designs of all of the studies conducted are briefly described in Table 1. The collection of analyzed milk samples described in **paper I** commenced in 2008, and samples were acquired from 39 farms throughout Estonia. The study lasted for two years and was part of a larger study within the framework of the Bio-Competence Centre of Healthy Dairy Products LLC, investigating the genetic parameters of milk coagulation properties in Estonia (Vallas *et al.*, 2010). The study described in **paper II** was carried out on the Eerika Experimental Farm of the Estonian University of Life Sciences (Märja, Estonia) from October 2009 till April 2010. The experimental farm was also used to conduct both a replicated 4 x 4 Latin square feeding trial in 2009, presented in **paper III**, which was part of a larger study (Kass *et al.*, 2012) based on milk samples collected, and observations of whole lactations for one year, from February 2011 till February 2012, as described in **paper IV**. Information on the days in milk, and calving dates of the cows were obtained from the Estonian Animal Recording Centre. All the experiments (**I, II, III,** and **IV**) were carried out according to the requirements of the Estonian Animal Protection Act.

Table 1. Experimental setup

Paper no	Milk samples	No of samples	Milk analyses	Additional analyses
I	Individual cow milk samples, collected as part of regular monthly animal recording. Special emphasis on non- and well-coagulating milk	143	Milk composition, pH, RCT, E_{30}, MS analyses	
II	Individual cow milk samples were collected twice a week for the first 100 days of lactation	133	Milk composition, pH, MS analyses	MS analyses of collected blood samples (n = 133)*
III	Individual cow milk samples from eight primiparous cows were collected at the end of each treatment period during a 4x4 Latin square feeding trial, where barley meal was replaced with crude glycerol	64	Milk composition, pH, RCT, E_{30}, MS analyses	Blood samples analyzed for insulin and glucose.* Rumen VFA content was analyzed from four cows
IV	Individual cow milk samples were collected as part of regular monthly animal recording throughout lactation	306	Milk composition, pH, RCT, E_{30}, MS analyses	

* Results not discussed in this thesis.

4.1.2. Milk samples

All test day milk yields were recorded, and in line with regular animal recording practices the milk samples were collected by in-line milk meters. The samples were stabilized with bronopol (Broad Spectrum Microtabs, D & F Control Systems Inc., Norwood, USA). Concentrations of the major milk compounds: milk fat, protein, SCC and urea, were measured at the Milk Analysis Laboratory of the Estonian Animal Recording Centre, with an automated milk analyzer (CombiFoss 6000, Foss Electric, Hillerød, Denmark). Before analyzing the coagulation ability, the pH of the milk was measured with a pH-meter (SevenMulti; Mettler Toledo GmbH, Greifensee, Switzerland).

4.1.3. Rumen fluid samples

Rumen fluid samples, as described in **paper III,** were collected from one of each of paired cows, which were fistulated with ruminal cannulas, at the end of each treatment period, and analysed for three volatile fatty acid (VFA) composition (propionic, valeric and isobutyric acids). An Agilent 7890A gas chromatograph (Agilent Technologies Inc, USA) with a 4% Carbowax 20M, matrix 80/120 Carbopack B-DA column (Sigma-Aldrich, St. Louis, USA) was used to measure the proportions of acids as described by Cottyn and Boucque (1968).

4.2. Coagulation analysis

For all milk samples, except for the 133 milk samples collected from October 2009 till April 2010 (**II**), rennet coagulation ability was measured. Both milk coagulation parameters, RCT (min) and E_{30} (mm), were determined with an Optigraph (Alliance Instruments, Frepillon, France) as described by Kübarsepp *et al.* (2005a) at the Laboratory of Milk Quality at the Estonian University of Life Sciences. Samples representing 39 farms in Estonia (**I**) were classified according to curd firmness. Milk which did not form a curd was classified as non-coagulating ($n = 27$, $E_{30} = 0$ mm) and well-coagulating milk had particularly good coagulation ability ($n = 27$, $E_{30} \geq 40$ mm). The remaining samples were divided into two groups: poor and good. If some curd firmness ($E_{30} > 0$ mm) was observed, the sample was classified as poor, and milk with good coagulation ability did form a firmer curd than poor, but the E_{30} value did not exceed 40 mm. A separate SCC-corrected database was made for milk samples with extreme coagulation ability, comprising samples with SCC $< 5 \times 10^5$ cells/mL. The rest of the analyzed milk was not divided according to coagulation ability (**III, IV**).

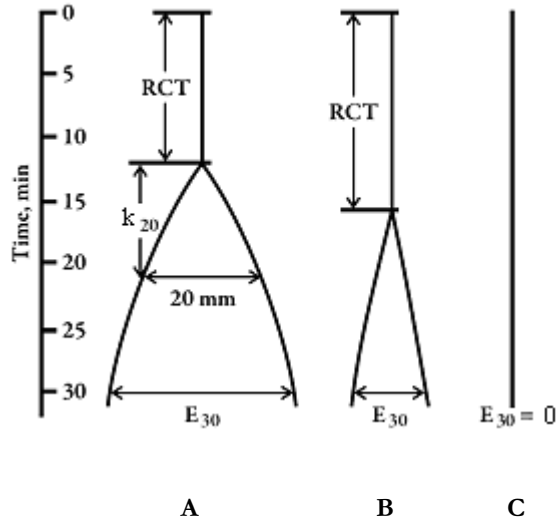


Figure 1. Coagulation graphs from the Optigarph and the Formograph representing well (A), poorly (B) and non-coagulating milk (C); showing common coagulation parameters such as rennet coagulation time (RCT), the time required to form a curd (k_{20}), and curd firmness in mm after 30 min of enzyme addition (E_{30}) (Kübarsepp *et al*., 2005b).

4.3. Cows and dietary treatment

In **paper I** data of the dietary rations of the two different breeds; Estonian Holstein (EHF, n = 121) and Estonian Red (ER, n = 22) are not known. Data from the Eerika Experimental Farm of the Estonian University of Life Sciences (Märja, Estonia) are well recorded, where the practice is to keep the cows in loose housed all year-round and are fed total mixed rations (TMR) *ad libitum* and milked before feeding twice a day (**II, IV**). The experimental farm used three different rations: (1) DM consisting of 40% of silage, 58% of concentrates and 2% of minerals, at 160 g kg^{-1} crude protein (CP), 103 g kg^{-1} metabolisable protein (MP) and 11.3 MJ metabolisable energy (ME); (2) DM 48% silage, 50% concentrates and 2% minerals, 159 g kg^{-1} CP, 97 g kg^{-1} MP, and 10.7 MJ ME; and (3) DM 73% silage, 25% concentrates and 2% minerals, 143 g kg^{-1} CP, 85 g kg^{-1} MP, and 9.7 MJ ME. The base ration comprised a grass and clover silage (75:25), barley, wheat and maize meal, heat-treated rapeseed cake, limestone, sodium chloride, and a vitamin-mineral mix designed for lactating cows. For the first 14 days after calving, the cows were fed ration (2), then ration (1) was offered at up to 6.5 months of lactation, or

when the milk yield remained at over 30 kg per day. Henceforward ration (2) and, one month before drying off, ration (3) was offered. In **paper II** five EHF cows were analysed. The whole lactation of 82 primi- and multiparous dairy cows (n = 156 and 150, respectively) of the EHF (n = 70), ER (n = 7) and Estonian Native (EN, n = 5) breeds was described in **paper IV**.

In **paper III** the feeding trial was carried out on eight primiparous EHF dairy cows. The cows were fed with TMR twice a day, and were milked before feeding. The base ration contained grass silage, barley meal, soya meal, limestone, sodium chloride and a lactating cow mineral mix (Table 1 in **paper III**) and was in accordance with Estonian feeding recommendations (Vabariiklik Söötmisalase Uurimistöö Koordineerimise Komisjon, 1995). During the experiment manipulations of the diet comprised alterations in the barley meal vs glycerol content, the control comprised barley meal and no crude glycerol (control; T0) and barley meal was substituted by crude glycerol in the following amounts: 1 (T1), 2 (T2) or 3 kg (T3). The isoenergetic balance of diets was maintained in all diets, the crude glycerol used had a metabolisable energy value of 14 MJ/kg (Mach *et al.*, 2009). Differences in crude protein were equalised with the addition of Optigen II (41.0% nitrogen, and 11.4% crude fat; Altech, USA), and all feed samples were analysed according to established methods (AOAC, 2005).

4.4. Mass spectrometric analyses

4.4.1. Challenges using mass spectrometry

It is important to keep variation among and between samples as minimal as possible, therefore sample collection (diurnal variation), storage (freezing, freeze raw or skimmed milk, time of sampling), stability (use of preservatives), pre-treatment (skimmed milk, precipitation of proteins) and instrument tuning were kept the same for all milk samples during all the experiments. Despite keeping variations minimal, the variation between different cows' milk samples were observed, which could be explained by differences in feeding, health and genetic factors. Generally, according to Scalbert *et al.* (2009), problems in the metabolic screening of milk may also be due to a chosen sample's preparation, separation (metabolites can be lost), ionization method and physicochemical

properties of different metabolites. There is not only one detector available to measure all metabolites, because some metabolites do not ionize, or their concentration is too low for detection. The experimental design chosen, using different MS-based approaches, may counter some problems, such as in fingerprinting standards, the quantification of unknown metabolites detected is complicated, or in some cases remains impossible (Scalbert *et al.*, 2009).

4.4.2. Milk treatment and mass spectrometric analyses

All collected milk samples were stored at −20 °C until analyzed for metabolites (**I, II, III,** and **IV**). Before further chemical analyses the frozen samples were thawed in a water bath at a temperature of 40 °C and, to remove the fat, a 5 mL aliquot of milk was centrifuged (Refrigerated Centrifuge 2-16K, Sigma-Aldrich, St. Louis, USA) at 3,000 × g for 10 min, at 4 °C. The resultant skimmed milk was mixed in equal volumes with a 3% trichloroacetic acid solution (ACS reagent, ≥ 99.5%, Sigma-Aldrich, St. Louis, USA) and centrifuged at 17,000 × g for 15 min, at 4 °C. The resulting supernatant was mixed again with an equal volume of acetonitrile (LC-MS CHROMASOLV, ≥ 99.9%, Sigma-Aldrich, St. Louis, USA) and centrifuged (Hettich Zentrifugen Universal 32R, GMI Inc., Ramsey**,** USA) at 14,000 × g for 15 min, at 4 °C. The supernatant was collected and mass spectrometry analyses where carried out.

MS analyses (**I, II, III,** and **IV**) were carried out on a triple quadrupole linear ion trap hybrid LC-MS/MS (3200 Q TRAP; AB Sciex Instruments, Foster City, USA) with an SIL-20A autosampler (Shimadzu, Kyoto, Japan) and LC-20AD pumps (Shimadzu, Kyoto, Japan). The mobile phase comprised of 80% acetonitrile in water and 0.1% formic acid. Positive and negative ionisation was obtained at room temperature (fingerprinting) or at 200 °C (targeted analyses) by Turbo Ion Spray. Samples were scanned in enhanced mode (EMS; fingerprinting; **I, II, III, IV**), and Multiple Reaction Monitoring mode (MRM; targeted metabolomics; **I, II,** and **III**), within the mass to charge (m/z) range of 50 to 1,500, and a scan rate of 1,000 amu/s. During fingerprinting, curtain and nebulizer gases had settings of 10 and 20, respectively, but during targeted analyses the settings were at 10 and 5, respectively. The Turbo Ion Spray voltage was set at 4,500 V. When the entrance and declustering potential, and the collision energy, were set at 10 V during fingerprinting, then for targeted analyses the entrance and declustering

potential, and the collision energy, were set at 20, 10 and 2 V, respectively. The preliminary compound identification in **papers I, II,** and **III,** was conducted on C_{18} (Luna 3 μm C18 100A, 100 x 2.00 mm, Phenomenox, Torrance, USA) and HILIC (Luna 5 μm HILIC 200A, 150 x 3.00 mm, Phenomenox, Torrance, USA) columns. For analyses of set retention time, the following gradient was used: 5 min isocratic at 95% acetonitrile in water, gradiental decline to 5% acetonitrile in water within 40 min, 10 min at 5% acetonitrile in water. For specific citric-acid cycle compound identification in **paper III** the gradient for retention time analyses was: 5 min isocratic at 95% ammoniumformate in methanol, gradiental decline to 5% ammoniumformate in methanol within 15 min, 5 min at 5% acetonitrile in water. All standard solutions and reagents for MS analyses were purchased from Sigma-Aldrich, St. Louis, USA, and the 3200 Q TRAP was controlled by the Analyst 1.4.2 software (Applied Biosystems Inc., USA).

For global analyses, and for metabolomic identification (**I, II, III,** and **IV**), mass to charge ratios of significant compounds were compared with the potential compounds in databases, including the MassBank (http://www.massbank.jp/), the Human Metabolome Database (HMDB; http://www.hmdb.ca/) and the METLIN Metabolite Database (http://metlin.scripps.edu/).

4.5. Statistical Analysis

Before any analyses were conducted on the MS spectra, the data were pre-processed by binning data to atomic mass unit resolution (**I, II, III,** and **IV**). In **paper I** the statistical significance between m/z intensities in the spectra of non-coagulating and well-coagulating milk was determined by Student's t-tests, and significant correlations were found using Pearson correlation analyses. Results were assessed with principal component analysis (PCA) and heat-map analyses, displaying the distributions of signal intensities relative to the mean at respective m/z values. M/z values were ranked based on calculated statistical differences, and only significant values were selected for presentation. In **paper II** the statistical significance between m/z intensities in the spectra of early and late lactation milk were determined by Student's t-tests, and results were displayed on a volcano plot, with the distribution of signal intensities relative to the mean at respective m/z values. M/z values

were ranked based on statistical differences, and only the significant values were chosen for presentation. PCA, heat-map and volcanoplot analyses (**I, II**) were conducted using R 2.8.1 BioConductor algorithms (R Development Core Team, 2009).

The model to test the statistical significance of treatment effect in the feeding trial (**III**) was as follows:

$$y_{ijkl} = \mu + D_i + P_j + C_k + e_{ijkl,}$$

where y_{ijkl} is the dependent variable, μ is the model intercept, D_i is the diet effect (i = 1, …, 4), P_j is the period effect (j = 1, …, 4), C_k is the random cow effect (k = 1, …, 8), and e_{ijkl} is the model error. Single treatments were compared according to their least square means. The associations between different VFA, blood and milk metabolites, and milk metabolic profile were assessed with Spearman partial correlation coefficients adjusted for the period effect.

To simultaneously examine the connectivity patterns of milk coagulation groups and mass spectrometry signals partial least squares correlation (PLSC) analysis was applied. PLSC is a multivariate statistical technique particularly well suited to situations where multicollinearity exists in the dataset, and the number of variables is high compared to the number of observations (Krishnan *et al.*, 2011). In this study, the connectivity patterns (also called latent factors) of three dummy variables of milk coagulation groups stored in matrix Y and 1900 standardized mass spectrometry signals (mass range under 1,000 Da in positive and negative ionization) stored in matrix X were evaluated by singular value composition of the form Y'X = USV' (the apostrophe denotes the matrix transposition). Matrices U and V are the matrices of the left and right singular vectors (representing the coagulation profiles and mass spectrometry signals profiles, respectively), best characterizing the correlation between X and Y; matrix S contains the singular values measuring the quality of latent factors. The percentage of variation accounted for by latent factor i was evaluated as the ratio of the sums of squares of latent variables (or scores) and initial variables: R^2_{Xi} = SS(XV_i)/SS(X) and R^2_{Yi} = SS(YU_i)/SS(Y) for mass spectrometry signals and coagulation, respectively; V_i and U_i denote the *i*th column of the matrices V and U. To test the statistical significance of latent factors the permutation test, with 1,000

permutation samples, was applied (to correct for the axis rotations and reflections the Procrustes rotation was used). This permutation also served to assess the singular vectors, giving a threshold to decide which variables were contributing the most to the latent factor.

Milk metabolome alterations during lactation were studied in **paper IV**. The identified principal components from PCA analyses were modelled following the general linear mixed model (GLM) as follows:

$$y_{ijklm} = \mu + B_i + P_j + D_k + M_l + b_1 \times LDIM_{ijklm} + b_2 \times LDIM^2_{ijklm} + b_3 \times LDIM^3_{ijklm} + C_m + e_{ijklm},$$

where y_{ijklm} is the dependent variable, μ is the model intercept, B_i is the breed effect (i = 1, 2, 3), P_j is the parity effect (j = 1, 2; primi- and multiparous), D_k is the diet effect (k = 1, 2, 3), M_l is the milking time effect (l = 1, 2; morning and evening), b_1*LDIM + b_2*LDIM^2 + b_3*LDIM^3 is the 3rd order Lagrange polynomial of days in milk, C_k is the random cow effect (k = 1, …, 82), and e_{ijklm} is the model error. The same model was fitted to milk coagulation, production and composition traits. To examine how the milk coagulation properties are related to the milk metabolome at different lactation stages, the lactation was divided into three parts: (1) the first sixty days of lactation referred as the beginning of lactation, (2) the 3rd to the 8th lactation months as the middle of lactation and (3) the last sixty days as late lactation. For each these time periods Spearman rank correlation analysis between m/z intensities in the spectra and milk coagulation properties was performed.

Statistical analyses applying PLSC and correlation analyses, and the modelling were performed with SAS software (version 9.1; SAS Institute Inc., Cary, NC, USA).

5. RESULTS

5.1. Development of methodology to analyse the milk metabolic profile

Sample preparation and MS parameters were optimized. The main criterion was high intensity of signals and low background noise throughout the scan range obtained in the spectra. Before acquiring the metabolic profile of milk by MS, the colloidal particles and high-molecular weight biopolymers were removed. In order to make fat removal easier, and to reduce losses, centrifugation was conducted at 3,000 × g for 10 min, at 4 °C (Refrigerated Centrifuge 2-16K, Sigma-Aldrich, St. Louis, USA). Centrifugation conducted at room temperature resulted in a soft and not easily removable layer of fat. To precipitate proteins a 3% trichloroacetic acid (TCA) solution (ACS reagent, ≥ 99.5%, Sigma-Aldrich, St. Louis, USA) and centrifugation at 17,000 × g for 15 min, at 4 °C, was used. As an alternative method to precipitate protein sodium dodecyl sulfate and chloroform was used (Tiemeyer *et al.*, 1984), but this method was too time-consuming for use constantly on a large number of samples. Before MS analyses the supernatant was mixed with an equal volume of acetonitrile (LC-MS CHROMASOLV, ≥ 99.9%, Sigma-Aldrich, St. Louis, USA) and centrifuged (Hettich Zentrifugen Universal 32R, GMI, USA) at 14,000 × g for 15 min, at 4 °C.

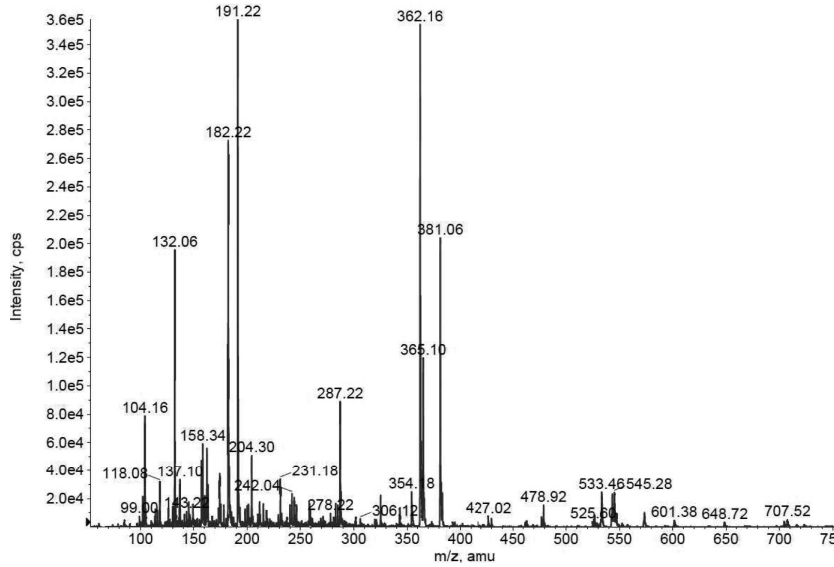
Optimizations of MS conditions for the multi-targeted analyses were first carried out manually. The curtain and nebulizer gases had settings varied from 0 to 50 and the Turbo Ion Spray voltage from -4,500 to -3,500 V for negative ion generation and 4,500 to 5,500 V for positive ion generation. Before the entrance and declustering potential, and the collision energy were set to 10 V, they had a range of -30 to 30 V. Additionally, ionization at temperatures 100 – 750 °C was tested. Elevated temperature was found to increase the signal from some compounds, most notably peptides and lipids. At the same time an increase in background noise and fragmentation of saccharides and organic acids was observed. In order not to lose the more fragile substances temperature elevation was not used in later studies.

5.2. Comparison of metabolic profiles of non- and well-coagulating milk

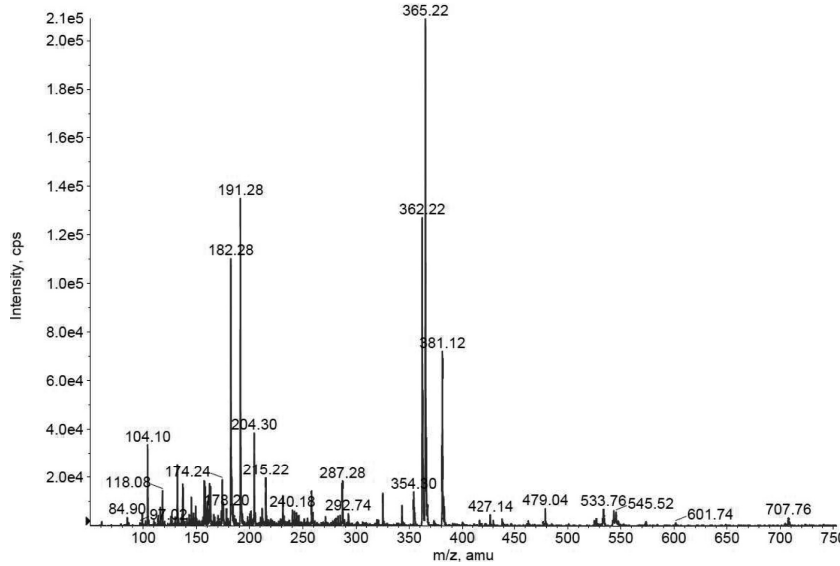
After the instrument tuning met requirements, and the sample pre-treatment steps were set, the first MS analyzes were preformed on 143 milk samples. At first the fingerprinting, analyses targeting no specific group of metabolites, was carried out and milk samples were scanned in the mass range of m/z 50 to 1,500. Typical MS spectra of non- and well-coagulating samples are visualised in Figures 2 A and B, respectively (**I**).

Although differences in the spectra of different milks were apparent, the role of biological variance and variance from other sources than coagulation needed to be explored.

Therefore to validate the hypothesis further, that the metabolic spectra of all non- and well-coagulating milk differs, the PCA was performed (Figures 3 A, B, and C). At first the metabolic spectra of all 143 milk samples were analyzed (Figure 3A) to assess if any prominent groups of metabolites would emerge visually. A distribution into well-defined clusters was seen when the intermediate samples were left out, and PCA was carried out only on non- and well-coagulating samples (Figure 3B). The same trend was observed when PCA was performed on an SCC-corrected database, where samples with a SCC over 5 x 10^5 cells/mL were omitted (Figure 3C). The total variation for all samples (Figure 3A) was caused by 91.2% of the components (comp 1 86%, and comp 2 5.2%).



A



B

Figure 2. Mass-spectrometric spectra of mass to charge ratio in the range of 50 to 750, of non- (A) and well-coagulating (B) milk.

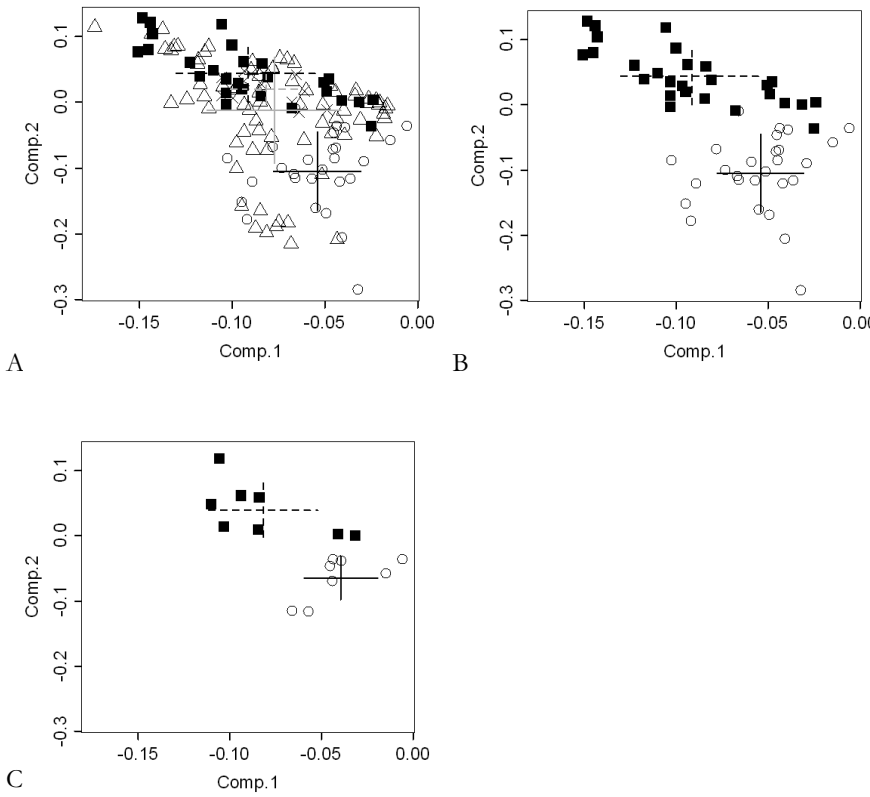


Figure 3. Principal component analyses: well- (▪), poor (Δ), good (×) and non-coagulating (○) samples, crosses represent the mean ± SEM; A) PCA of all 143 milk samples; B) as A, but only well- (▪) and non-coagulating (○) milk samples; C) as B, but only the SCC-corrected database was used.

Subsequently, heat-map analyses were conducted to display the distribution of signal intensities relative to the mean at respective m/z values. The m/z values were ranked based on the calculated statistical differences between the signal intensities in the non- and well-coagulating groups, and the 25 most significant differences are shown (Figure 4). Alterations in both directions were found: some compounds were more abundant in well-coagulating milk and others in non-coagulating milk, and the non- and well-coagulating milks were significantly different ($P \leq 0.05$). Compounds with m/z values 534, 601, and 162 represented on heat-map, were expressed more intensely in samples with better coagulation ability (Figure 3 A, B, and C in **paper I**).

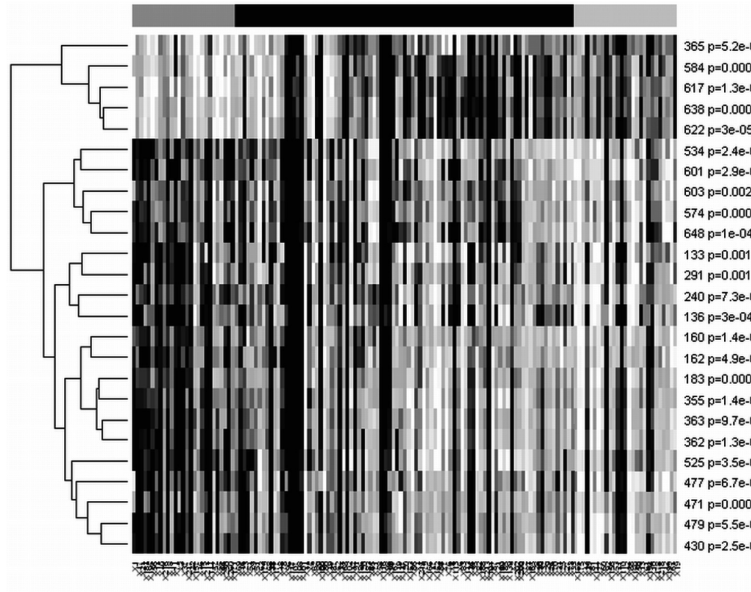


Figure 4. Heat-map of non- (dark gray panel on the left), intermediate (black panel) and well-coagulating (light gray panel on the right) milk samples. The 25 most significantly different m/z values calculated with the Student's t-test between well- and non-coagulating groups and results as p-values can be seen in the right hand column. The dendrogram on the left hand side has rows arranged into clusters based on pattern similarities. The lighter a data point is the more intense the signal is compared to the mean value for the respective m/z among all analyses: gray points are close to the mean and dark tones are below the mean.

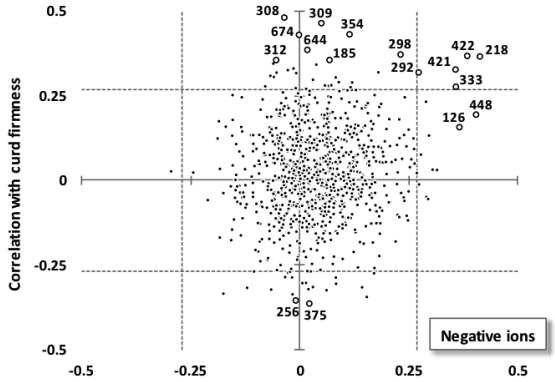
As hypothesized, mass spectrometric analysis of low molecular weight milk compounds can pinpoint metabolites which are correlated with coagulation ability. To identify those compounds targeted mass spectrometric analyses (e.g. fragmentation analyses; Table 3 in **paper I**) were conducted on those compounds visualized in Figure 4. The results from fragmentation analysis were compared with the spectra in the Human Metabolome Database (htttp://www.hmdb.ca) and the METLIN Metabolite Database (http://metlin.scripps.edu/), and only the signal of m/z = 162 was positively identified, as the spectra resembled that of L-carnitine. Unfortunately for the rest, no defined match was found, although it was noted that some spectra shared similarities between each other. Initially a repetitive loss of 18 Da was observed; next the fragments with m/z values of 99, 180 and 261 were shared by many compounds; and lastly the compounds with higher mass yielded fragments matching molecular weights of smaller

compounds. Therefore, it was noted that the fragmentation spectra imply oligosaccharides, and this was further tested by determining fragmentation and HILIC retention times of glucose, sucrose, lactose and N-acetyllactosamine (Table 3 in **paper I**). During the fragmentation analyses protonated molecular ions, sodium and potassium adducts and $[M\text{-}H_2O]^+$ ions were observed. Signal m/z = 365, was matched with the spectra of commercial N-acetyllactosamine (Sigma-Aldrich, St. Louis, USA) representing the $[M\text{-}H_2O]^+$ ion. Despite this positive identification, the results should be taken with caution because various saccharides have high structural similarities with each other. Four out of the five most significantly elevated signals in non-coagulating milk had a fragment of m/z = 365 in their spectra.

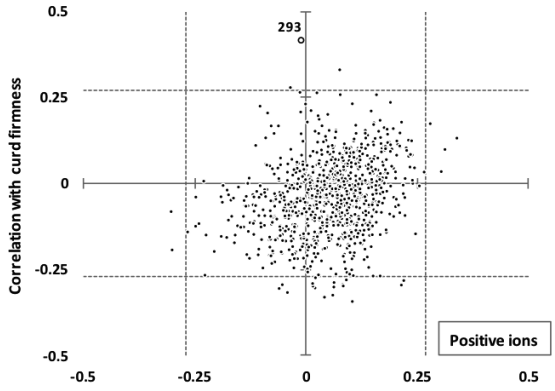
5.3. Effect of glycogenic precursors on milk coagulation and metabolites

As a result of the changes made to the TMR, where part of the barley meal fed was replaced with crude glycerol, a change in the proportions of glycogenic VFAs in the rumen was observed (Figure 1 in **paper III**). The proportion of propionic acid produced was significantly different from the control at T2 and T3 ($P = 0.030$, and $P < 0.001$, respectively), having a strong positive correlation with glycerol addition ($r = 0.71$, $P < 0.001$). The proportion of valeric acid was also significantly different between the control at T3 ($P = 0.030$).

Variations between different milk traits were not significantly different, except for protein concentration between all treatments ($P < 0.001$), and for pH between the control at T3 ($P = 0.010$). Mean milk curd firmness (E_{30}) improved linearly as the barley concentration in feed decreased and glycerol supplementation increased ($P < 0.001$), and was positively correlated with milk protein ($r = 0.58$, $P < 0.001$), propionic and valeric acid ($r = 0.38$ and $P = 0.07$; $r = 0.32$ and $P = 0.13$, respectively); and negatively correlated with isobutyric acid ($r = -0.56$, $P = 0.005$). To describe how the addition of crude glycerol to the diet alters the milk coagulation ability, Spearman partial correlation analyses were carried out (Figure 5).



A

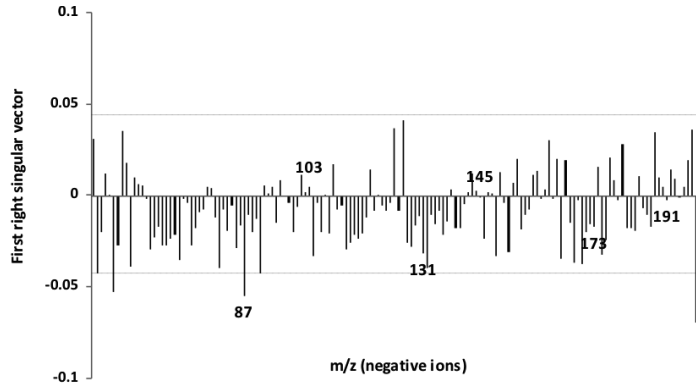


B

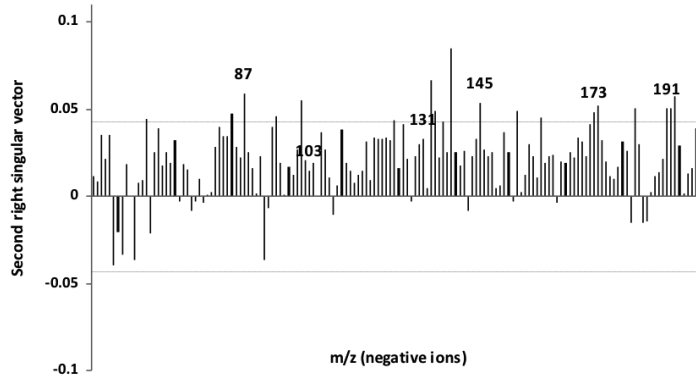
Figure 5. Spearman partial correlations (adjusted for period effect) of glycerol addition and curd firmness for masses measured in negative ion mode (A) and positive ion mode (B). Labelled circles correspond to correlations with $P < 0.01$ in the horizontal or vertical direction, or indicate the masses significantly ($P < 0.05$) correlated with both glycerol addition and curd firmness. Dashed lines denote the cut-off for statistical significance of correlation coefficients ($P = 0.05$).

Signals measured in positive ion mode had no signals that correlated with both glycerol addition and curd firmness, but one signal $m/z = 293$ (histidine and/or lysine) was associated with firmer curd. Measurement in the negative ion mode singled out the five most prominent signals $m/z = 218$, 422, 421, 333 and 292, associated with both curd firmness and crude glycerol addition. Fragmentation analyses of these compounds indicated the presence of glycerophosphocholine and panthothenic acid. To evaluate the energy profile of milk, the milk organic acid

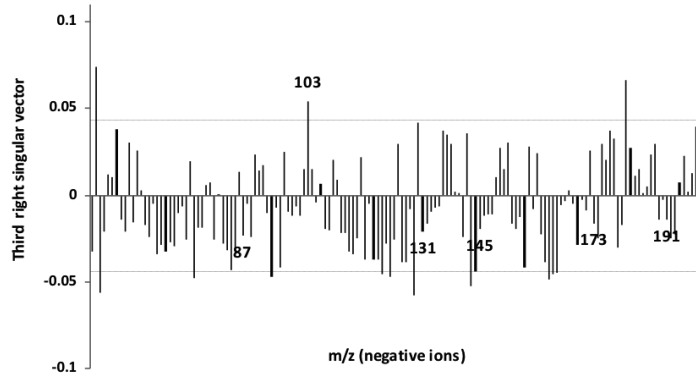
composition was analysed. Changes in citric-acid cycle component concentrations (e.g. citrate, pyruvate, cis-aconitate, α-ketoglutarate, malonate, and oxaloacetate; Table 2 in **paper III**) in treatment periods were analyzed. Malonic, oxaloacetic, pyruvic, and α-ketoglutaric acids were positively correlated with curd firmness (r = 0.25, $P = 0.06$; r = 0.33, $P = 0.013$; r = 0.13, $P = 0.34$; and r = 0.11, $P = 0.42$, respectively). No correlations were observed between citrate and cis-aconitate with curd firmness (r = -0.01, $P = 0.94$, and r = 0.02, $P = 0.88$, respectively). Partial least squares correlation analyses were conducted on citric acid cycle compounds relationships to feeding groups (Figure 6).


First right singular vector
0.1
0.05
0
-0.05
-0.1
103
145
191
173
131
87
m/z (negative ions)

A


Second right singular vector
0.1
0.05
0
-0.05
-0.1
87
145
173
191
131
103
m/z (negative ions)

B


Third right singular vector
0.1
0.05
0
-0.05
-0.1
103
87
131
145
173
191
m/z (negative ions)

C

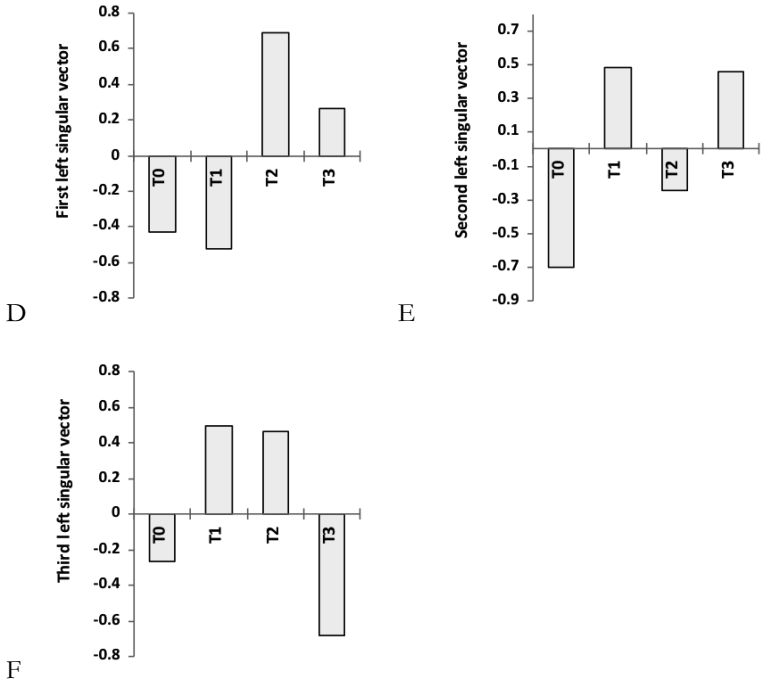


Figure 6. A, B, and C: Results of partial least squares correlation analyses. Peaks denote the strength and direction of the masses contributions in relation to the two latent factors. Signals shown represent citric acid cycle compounds (87 is pyruvic, 103 malonic, 131 oxaloacetic, 145 α-ketoglutaric, 173 cis-aconitic and 191 citric acid). Dashed lines denote the approximate cutoff for statistical significance of the right singular vectors (masses vectors) as assessed through permutation tests ($P = 0.05$). **D, E, and F:** Results of partial least squares correlation analyses. Bars denote the strength and direction of the feeding groups' contributions in relation to the two latent factors.

5.4. Alterations in milk coagulation ability and its relationship with the metabolome during lactation

On 306 milk samples GLM analyses were conducted and curves estimated for curd firmness and RCT showed changes during lactation (Figure 7; Table 1 in **paper IV**). Curd firmness maintained favourable values throughout the lactation, the lowest value was observed during midlactation (30.42 mm on the 157th day), and the best on the first and last 60 days (> 32 mm). Curves describing RCT showed an increase over time till the end of the midlactation period. During midlactation coagulation time was the longest (day 172-238, maintaining a mean value

of between 9.90 and 9.99 min) and shortest in the first 18 days (less than 6 min).

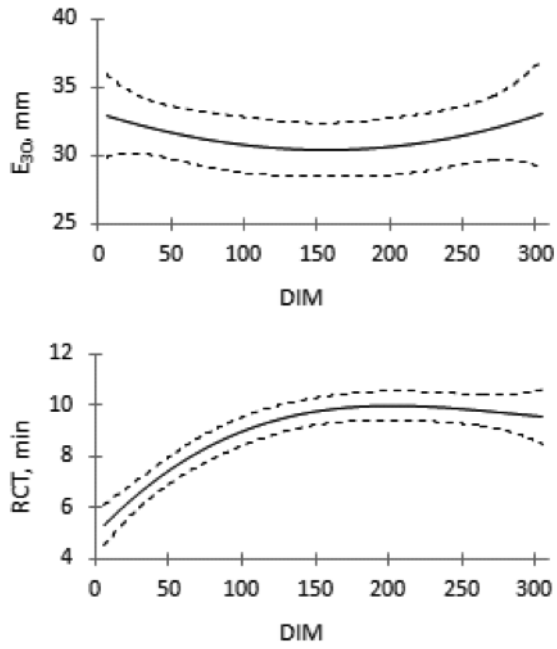
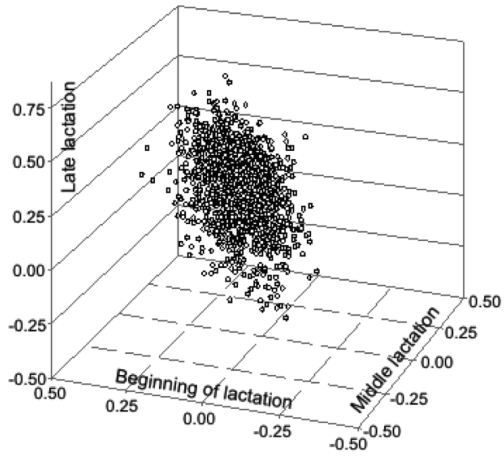
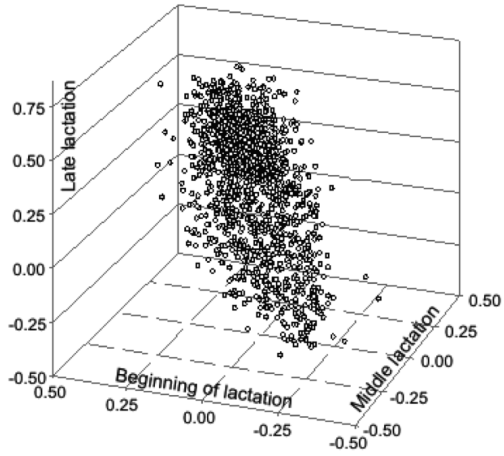


Figure 7. Model of curd firmness (E_{30}) and milk coagulation time (RCT) (with 95% confidence interval) during whole lactation, estimated curve with 3rd order polynomial according to the linear mixed model. Fixed effects of breed, parity (primi- and multiparous), diet and milking time (morning or evening) and random effect of cow, have been considered.

Modelled dynamics describing the change during lactation were strongly animal-specific (Table 1 in **paper IV**) and traits such as curd firmness were influenced by milking time; a firmer curd and shorter RCT were observed at evening milking. To verify relationships between milk coagulation properties and the milk metabolome at different lactation stages, Spearman rank correlation coefficients were calculated (Figure 8). Although no strong relationships were found, a trend was calculated that was similar across all three periods. The intermediate positive relationship (correlation coefficient $r > 0.3$) with E_{30}, at the beginning, mid and late lactation, could be related to two signals in positive ion mode (m/z = 197 and 342) and eight in negative ion mode (m/z = 612, 737, 835, 836, 902, 1000, 1038 and 1079). The RCT had no signals with at least an intermediate relationship during all three lactation stages.



A



B

Figure 8. Spearman rank correlation coefficients between signals measured at both positive (A) and negative (B) ionisation and curd firmness (E_{30}). Dots correspond to signals measured with MS representing signals correlations with E_{30} during the three different lactation stages.

6. DISCUSSION

6.1. Assessment of milks' metabolic profiles

To get a snapshot describing the small molecules present in a cell, organ or organism of interest at a specific time, the metabolic profile has to be measured. The measurement can be carried out on different instruments, depending upon what is looked for. As the aim was neither to look for volatile compounds nor carry out derivatisation (O'Mahony *et al.*, 2013), profiling was carried out on an LC-MS/MS not on a GC-MS. To test the hypothesis that non- and well-coagulating milk samples have different metabolic profiles, a method to assess the metabolic profile had to be devised. The aim was to keep the pre-treatment of milk samples as time- and cost-effective as possible. As the second aim was to describe profiles of low molecular weight compounds it was desirable to extract the entire set of these from milk proteins. Proteins themselves are too large to be analyzed by triple quadrupole type MS apparatus, and they cause an increase in background and reduced signal-to-noise quality. In addition, electrospray ionization is not well suited for the analysis of colloidal systems. The presence of lipid micelles leads to signal instability and possible clogging of the apparatus's capillary systems. Therefore peptides and neutral lipids are not easily visualised, and thus the methodology used is biased towards easily ionized compounds.

The most cost-effective, and standard, method to remove fat from samples is centrifugation at a low temperature. Trichloracetic acid (TCA) is widely used to precipitate protein in milk, but it is usually used at higher concentrations (Furusawa, 1999). As the MS is sensitive to background noise, the TCA concentration was kept low, and additional protein precipitation was carried out with acetonitrile. TCA and acetonitrile precipitation of plasma proteins has previously been studied by Cheng *et al.* (2010). The resultant supernatant was collected and introduced into the mass spectrometer via an auto sampler for further analyses.

The pre-treatment of milk samples developed, and instrument tuning, as discussed in section 4.4.2. allowed the achievement of mass spectra of 646 milk (**I, II, III,** and **IV**) and 133 blood (**II**) samples measured in positive and negative ion modes. Results from blood analyses are outside the scope of this thesis and are not reviewed further. Determining the

metabolic spectra, and comparing the spectra of samples with different coagulation ability, feeding or lactation stage, helps develop further the potential for marker-assisted pre-selection of milk suitable for cheesemaking. As the factors limiting cheese yield are of interest, these analyses were conducted to determine if there are any extant underlying metabolites for milk coagulation.

6.2. Alterations in metabolic profile

The experimental design covered three different aspects of metabolic profile measurements. First, the basis for measuring the metabolome of milk was set, and carried out on 276 samples. The differences in spectra were observed, depending on coagulation ability (**I**) and days in milk (**II**). Therefore the effect of lactation stage on coagulation ability (**IV**) and its relationship with the metabolic profile was investigated in further detail, and as data about the effect of feeding on milk biomarkers was scarce, an additional study investigating the effect of feeding was devised (**III**).

6.2.1. Coagulation ability

As cheesemaking starts with the selection of milk, the milk has to match appropriate criteria, e.g. good coagulation ability and high protein content are preferred (Bittante *et al.*, 2012). The coagulation ability of bovine milk has been widely studied (Ikonen *et al.*, 1999; Cassandro *et al.*, 2008; Vallas *et al.*, 2010; Bittante *et al.*, 2012), and although many factors (e.g. breed, feeding, protein genotypes) affecting milk coagulation ability have been determined (Schaar, 1984; Cassandro *et al.*, 2008; Jõudu *et al.*, 2009; **paper III**), the influence of the metabolic profile on coagulation ability is still under investigation (Sundekilde *et al.*, 2011; **paper I**). The objective of the first study was to determine the metabolic profiles of non- and well-coagulating milks, and, as hypothesised, the differences in the metabolic profiles of these two groups were observed (**I**). Global metabolic analyses of metabolites in the mass range m/z 50 to 1,500 showed that some metabolite intensities were correlated with coagulation ability (Figure 2). There were not only one or two metabolites associated with a difference, but many more. As seen from PCA (Figure 3A, B, and C) the first two components represented the total variation in 91.2% of all compounds found in the metabolic profiles of the analysed milk samples. Besides non- and well-coagulating milk samples, intermediate

samples were also analysed. Both from PCA (Figure 3A) and a heat-map (Figure 4), samples with poor coagulation ability were more similar to non-coagulating milk, whereas the others, with better coagulation ability, were more similar to the well-coagulating milk samples. A positive effect of elongating the coagulation time from 30 to 45 min was observed by Cecchinato *et al.* (2013), where 6.67% of milk samples did not coagulate within a period of 30 min. This could indicate by extending the RCT more of the samples would have had time to coagulate and would have been recorded as coagulating milk.

According to Politis and Ng-Kwai-Hang (1988) the cheesemaking process is significantly affected by the SCC. An elevated level of SCC is usually linked to mastitis and has an unfavourable effect on coagulation ability, because of the altered milk protein and fatty acid proportions, and increased enzymatic activity (Politis *et al.*, 1989; Ogola *et al.*, 2007; Sharif, Muhammad, 2008). Therefore, in the first study (**I**) the SCS (SCS = log SCC) was subjected to correlation analysis with the MS signals. Overall general correlation was found to be weak ($r = 0.28$), and no significant correlation with SCS was found for the lower mass range, under m/z 499, but the most positive correlation was with signals with m/z over 1,000. On analysing the results of correlation analyses further, it was observed that a high SCC causes higher background noise from peptides and oligosaccharides. The SCC was taken into account by performing both PCA and heat-map analyses. As predicted, even when the SCC-corrected database was used, the samples of non- and well-coagulated milks formed distinct groups (Figure 3B and C) and signal intensities showed the same trend (Figures 3A and C in **paper I**). This therefore indicates that the processes carried out at the molecular level do not depend only on the somatic cell count.

Subsequently, to further validate the hypothesis and provide a closer understanding of the mechanism behind unsatisfactory coagulation, the identification of significantly different metabolites visualized on a heat-map (Figure 4) was carried out. Compounds measured in positive mode with $m/z = 534$, 601, and 162 were particularly interesting, because the signals were more intense in samples with better coagulation ability ($P \leq 0.05$) and are represented on all heat-maps (Figures 3A, B, and C in **paper I**).

The major limiting factor during these studies was the identification of metabolites. Of the significant signals only one definite match was found, L- carnitine. According to Harmeyer (2002), L-carnitine, also known as vitamin $B_{T,}$ is a naturally occurring constituent in muscle, plasma and milk. L-carnitine is considered to be an essential nutritional factor with a function as fatty acid (> C_{14}) carrier in mitochondria, and it also contributes to inhibition of lipolysis. The concentration of L-carnitine in milk (0.02 to 0.04g L^{-1}) is tenfold higher than in plasma, but 10 to 30 times less than in muscle. L-carnitine content in milk is related to lactation stage and number, as well as to the milk yield; it declines significantly during lactation, with cow age and with higher milk yield (Harmeyer, 2002). According to Harmeyer (2002) the decline may, with caution, be considered as a state of deficiency of L-carnitine. Thus, better coagulation was observed in nutritionally balanced samples.

Other spectra had some similarities with each other, as the repetitive loss of 18 amu, fragments with m/z = 99, 180, and 261 were common to many compounds, and the compounds with higher masses yielded fragments that matched the molecular weights of smaller compounds; implying the fragment spectra to be oligosaccharides. Bovine milk glycome has been widely studied, and natural variability has been observed (Urashima *et al.*, 2001; Tao *et al.*, 2008; Tao *et al.*, 2009; Sundekilde *et al.*, 2012). Keen interest in oligosaccharides is due to their nutritional value to neonates and intestinal bacteria (Gopal, Gill, 2000; Tao *et al.*, 2008). Associations between oligosaccharides and the technological properties of milk have not been reported previously, although the unfavourable effect of polysaccharides on coagulation ability has been noted (Olsen, 1989; Pires *et al.*, 2004). Further testing of the signal of interest implied the signal m/z = 365 matched fragments from commercial *N*-acetyllactosamine (Sigma-Aldrich, St. Louis, USA). The fragment with m/z = 365 was observed in all of the four signals that were elevated in non-coagulating milk, but none in well-coagulating milk. *N*-acetyllactosamine can be found in milk in a complex with lactose (Bode, 2006) or as part of the milk 689-long amino acid residue lactofferin, which also includes saccharides such as *N*-acetylglucosamine, fucose, galactose, mannose and neuraminic acid (Steijns, van Hooijdonk, 2000). Although bovine milk has fewer different oligosaccharides than human milk (Tao *et al.*, 2009), the positive identification of these with MS could still be difficult, as the structures of various saccharides are similar. It would be better

to use NMR for this kind of analysis, as structural identification can be made. But as bovine milk contains oligosaccharides in low amounts (Gopal, Gill, 2000), and for NMR the compound of interest needs to be abundant, this method is inappropriate. Fortunately, MS methods used to carry out oligosaccharide identification are improving (Ruhaak, Lebrilla, 2012). Thus this identification in **paper I** should be regarded with caution. Further analysis should be conducted to determine the exact oligosaccharides affecting coagulation ability.

For the rest of the signals in the metabolic profile, the molecular weights were calculated according to the values of charges known, and most were observed to be above 500 amu. According to Kind and Fiehn (2007) nonpolymeric compounds rarely exceed a molecular weight of 600 amu, and markers around m/z 1,000 could indicate lipids, peptides, or oligosaccharides.

6.2.2. Feeding

The usual practice in Estonia is to feed barley grain to dairy cows as part of a TMR (Table 1 in **paper III; IV**). Barley has a high starch content (Waldo, 1973), and it is extensively (up to 90%) fermented in the rumen by microbial fermentation, and as result organic acids are produced (Huntington, 1997; Larsen *et al.*, 2009). Rapidly degradable starch is used for energy as glucose by microbes, or as volatile fatty acids (VFA) by cows (Huntington, 1997). VFA occurrence is associated with diet composition, milk yield and composition (Murphy *et al.*, 1982; Dijkstra *et al.*, 1993). During starch fermentation propionate, a glycogenic precursor, is produced in abundance. This is a favourable product, as it is converted into glucose in the liver. But high blood propionate concentrations are not desirable, because it increases insulin production, and high insulin production increases the uptake of nutrients in different tissues, which in turn affects lactation by the reduction of lipolysis, thus decreasing milk production and milk fat content (Ørskov, 1986). As the high starch content results in a high, unfavourable, propionate content, the possible partial replacement of barley meal with crude glycerol was investigated. Glycerol, a by-product of biodiesel production, was for many years considered as a feed additive and was used as an alternative energy source for dairy cows, or as a prophylactic treatment for ketosis (DeFrain *et al.*, 2004). It is now regarded as a good energy source for livestock, and a safe feed for animals. As studies describing the effect of crude glycerol

on low molecular compounds of milk and coagulation ability are scarce, the emphasis in **paper III** was on coagulation and MS analyses of milk.

As stated previously, overall differences during the feeding trial between different milk traits were not significant, except for the milk protein concentration between all treatments, indicating a faster outflow of microbial protein from the rumen (Pathak, 2008), which increases the production of amino acids; the building blocks of milk protein. The increased protein content was positively correlated with curd firmness ($r = 0.58$, $P < 0.001$), which in turn indicates a positive affect of replacing barley meal with crude glycerol in diets on milk coagulation ability. The positive effect of milk protein concentration on milk coagulation ability has also been observed by Jõudu *et al.* (2008).

In addition to the protein content, positive correlations between curd firmness and two of the glycogenic precursors - propionic and valeric acid ($r = 0.38$ and $P = 0.07$; $r = 0.32$ and $P = 0.13$, respectively) were found, while the third glycogenic precursor, isobutyric acid, had a strong negative correlation with curd firmness ($r = -0.56$, $P = 0.005$). The overall proportions of glycogenic precursors changed with an effect of treatment, being significantly different from the control during treatment periods when barley meal was replaced with 2 and 3 kg of crude glycerol. Thus, the milk coagulation ability could be related to the change in the rumen VFA proportions, which may be a result of a change in the microbial population.

It was also hypothesized that a change in the glycogenic precursor alters the milk metabolic profile. Therefore mass spectrometry analysis and identification of milk metabolites was conducted, to get a better understanding of the reasons for the change in coagulation ability. A change in the metabolic profile for milk samples with different curd firmness was correlated with the supplementation of crude glycerol. From the recorded signals in positive mode, only one prominent signal $m/z = 293$ appeared. This was positively correlated with curd firmness, while crude glycerol had no effect. Fragment spectra indicated the presence of two amino acids – histidine and lysine. The abundance of both of these amino acids in the well coagulating milk samples is in accordance with the increased protein concentrations, as histidine and lysine, and also methionine, are limiting amino acids for milk protein production (Kim *et al.*, 2001).

At negative ionization, the number of significantly different signals was high, and the most significant signals were representative of both good coagulation and the change in the glycogenic precursor in the diet, were m/z = 218, 422, 421, 333 and 292 (Figure 5B). Fragmentation analyses carried out on signal m/z = 292 indicated fragments matching the spectra of glycerophosphocholine. The glycerophosphocholine to phosphocholine ratio is used as a biomarker for ketosis, where higher glycerophosphocholine values indicate a low risk of ketosis (Klein *et al.*, 2012). Signal m/z = 218 indicated fragments of the spectra of pantothenic acid. Panthothenic acid is a component of ruminants feed and it is synthesised by the rumen microbes, hence the synthesis of pantothenic acid depends on the diet, and increases as the supplementation of rapidly degradable carbohydrates is increased (Ragaller *et al.*, 2011). Pantothenic acid is associated with the energy metabolism of cells, as it is usually bound to Coenzyme A (Ragaller *et al.*, 2011). No positive effect of panthothenic acid on milk coagulation has previously been reported, but in the current study, it was positively correlated with curd firmness, and with a change in the glycogenic precursor in the diet.

As with other anions (e.g. carbonate, phosphate, lactate, acetate, chloride) citrate influences the physical-chemical conditions of milk (Salaün *et al.*, 2005) and therefore, by interacting with other milk constituents, it affects milk-processing quality (e.g. coagulation) (Garnsworthy *et al.*, 2006). As a common milk component, citrate is capable of buffering milk and, according to Visser *et al.* (1979), citrate ions can improve the binding of calcium and phosphate ions to casein micelles. As the name indicates, citrate is an important intermediate in the citric-acid cycle. It has a role in cellular energy metabolism, and thus is referred to as an indicator of cows' energy status (Baticz *et al.*, 2002). As reported by Linzell *et al.* (1976) citrate cannot pass through mammary epithelium, therefore it is produced in the mammary secretory cells in the udder. Milk citrate concentration is affected by lactation (Garnsworthy *et al.*, 2006) and feeding (Faulkner, Peaker, 1982). However, Gransworthy *et al.* (2006) have stated that bovine milk citrate concentration does not depend on milk yield, diet or mastitis. The difference in citrate concentration was observed at the beginning of lactation and was associated with the oxidation of fatty acids. Due to inconsistency in the literature about the impact of changes in the diet on the milk energy profile (e.g. milk organic acid composition), the changes in citric-acid cycle components' (e.g. citrate, pyruvate, cis-

aconitate, α-ketoglutarate, malonate, and oxaloacetate; Table 2 in **paper III**) concentrations between treatment periods were measured, and associations with coagulation ability were analysed. Only malonic and oxaloacetic acid were positively correlated with curd firmness (r = 0.25, $P = 0.06$, and r = 0.33, $P = 0.013$, respectively) and overall concentrations of milk citric-acid cycle components did not change significantly with treatment (Figure 6). Hence the partial replacement of barley meal with crude glycerol had no effect on the citrate concentration which is not directly associated with coagulation ability.

6.2.3. Stage of lactation

During lactation the milk composition tends to change, as the suckling calf grows and its nutritional needs change over time (Walstra, 1984). Maybe due to these biological changes coagulation ability, which has been widely investigated (Ostersen *et al.*, 1997; Summer *et al.*, 2003; Tyrisevä *et al.*, 2003) also changes over the lactation (Vallas *et al.*, 2010). It tends to be the best at the beginning, worst in the middle, and good at the end of lactation (Ostersen *et al.*, 1997; Summer *et al.*, 2003; Tyrisevä *et al.*, 2003). Therefore the metabolic profile of milk over lactation was observed and associated with coagulation ability.

The estimated curve for curd firmness maintained favourable values throughout lactation with no noticeable peaks (Figure 7; Table 1 in **paper IV**). This is in good accordance with the results of Ostersen *et al.* (1997), as the curd firmness was at its best (> 32 mm) at the beginning and the end of lactation. Low curd firmness was recorded during midlacatation (30.42 mm on the 157th day). Greater variation in curd firmness was observed by Vallas *et al.* (2010) than in the current study, but the trends of the curves describing RCT were consistent in the two studies. Another trait describing coagulation ability, the RCT, was best during the first 18 days (less than 6 min) and worst during midlacation (days 172-238, maintaining mean values of between 9.90 and 9.99 min). Towards the end of lactation the RCT decreased, reaching 9.55 min. Shorter RCTs at the beginning and at the end of lactation have also previously been noted by Ostersen *et al.* (1997). Many studies (Grandison *et al.*, 1984; Ostersen *et al.*, 1997; Kübarsepp *et al.*, 2005a) have previously associated the increase of RCT with an increase in pH. The low pH during the beginning of lactation in fat cows is associated with long-chain fatty acid synthesis and higher citrate concentrations (Ostersen *et al.*, 1997).

Furthermore, it was observed that milk coagulation traits were strongly animal specific (Table 1 in **paper IV**), and in addition to the effect of animal, curd firmness was influenced by milking time, a firmer curd and shorter RCT was observed during evening milking.

The correlations between milk coagulation properties and milk metabolome at different lactation stages were not strong, although the direction and strength of the correlations remained more or less the same at the beginning, middle and end of lactation (Figure 7). Only two signals during positive (m/z = 197 and 342), and eight in negative (m/z = 612, 737, 835, 836, 902, 1000, 1038 and 1079), ionization had a positive correlation (r > 0.3) with curd firmness at each lactation stage. For the RCT, no such signals appeared. This indicates that the change in E_{30} could be associated with fluctuations of some metabolites, as the RCT is not so metabolite-dependant.

7. CONCLUSIONS

- These results show that the robust method devised for the pre-treatment of milk samples (fat removal and protein precipitation) to analyse milk with mass spectrometry (LC-MS/MS) for low molecular compounds allows the acquisition of the milk metabolic profile. The detected signals (e.g. L-carnitine) can be associated with either coagulation ability or other parameters (e.g. SCC) believed to influence the coagulation process, or some other process taking place in the organism (**I, II**).

- The metabolic differences of milk with different technological properties, such as extremes of coagulation, can be observed by LC-MS/MS analysis. It is not yet possible to describe in detail the mechanisms whereby the metabolites identified (e.g. carnitine, panthotenic acid) in these studies enhance or inhibit coagulation, but the results presented here are a step in this direction (**I, III, IV**).

- Based on preliminary results a change in the glycogenic precursor in the diet can alter the milk metabolic profile. The milk coagulation ability may be improved through altering proportions of rumen VFAs, as well as milk protein and energy metabolite (e.g. citric-acid cycle components, pantothenic acid) concentrations. Therefore crude glycerol, as an alternative dietary source for glucogenesis, appears to have a favourable effect when fed to the dairy cow (**III**).

- It was demonstrated that there are metabolites specific to curd firmness throughout the lactation. The change in the metabolome had no correlation with RCT (**IV**).

Future studies comprise identification of the metabolites associated with lactation stage. As the metabolic analyses have been made on individual cow's milk, the bulk milk and cheesemilk should also be analysed. Therefore, the selection of good milk for cheesemaking could be improved, and metabolites, or sets of them could be associated with cheese yield.

REFERENCES

Antignac, J.-P., Cariou, R., Bizec, B.L, André, F. 2004. New data regarding phytoestrogens content in bovine milk. *Food Chemistry* 87, 275-281.

AOAC. 2005. Official Methods of Analysis of Association Official Analytical Chemists. 18th edition - Gaithersburg, MD, USA, Association Official Analytical Chemists.

Baticz, O., Tomoskozi, S., Vida, L., Gaal, T. 2002. Relationship between concentration of citrate and ketone bodies in cow's milk. *Acta Veterinaria Hungarica* 50, 253-261.

Bittante, G., Contiero, B., Cecchinato, A. 2013. Prolonged observation and modelling of milk coagulation, curd firming, and syneresis. *International Dairy Journal* 29, 115-123.

Bittante, G., Penasa, M., Cecchinato, A. 2012. Invited review: Genetics and modeling of milk coagulation properties. *Journal of Dairy Science* 95, 6843-6870.

Bjerre-Harpoth, V., Friggens, N.C., Thorup, V.M., Larsen, T., Damgaard, B.M., Ingvartsen, K.L., Moyes, K.M. 2012. Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different stages of lactation. *Journal of Dairy Science* 95, 2362-2380.

Blasco, C., Pico, Y., Andreu, V. 2009. Analytical method for simultaneous determination of pesticide and veterinary drug residues in milk by CE-MS. *Electrophoresis* 30, 1698-1707.

Bode, L. 2006. Recent Advances on Structure, Metabolism, and Function of Human Milk Oligosaccharides. *The Journal of Nutrition* 136, 2127-2130.

Boudonck, K.J., Mitchell, M.W., Wulff, J., Ryals, J.A. 2009. Characterization of the biochemical variability of bovine milk using metabolomics. *Metabolomics* 5, 375-386.

Cassandro, M., Comin, A., Ojala, M., Dal Zotto, R., De Marchi, M., Gallo, L., Carnier, P., Bittante, G. 2008. Genetic parameters of milk coagulation properties and their relationships with milk yield and quality traits in Italian Holstein cows. *Journal of Dairy Science* 91, 371-376.

Cecchinato, A., Cipolat-Gotet, C., Casellas, J., Penasa, M., Rossoni, A., Bittante, G. 2013. Genetic analysis of rennet coagulation time, curd-firming rate, and curd firmness assessed over an extended testing period using mechanical and near-infrared instruments. *Journal of Dairy Science* 96, 50-62.

Cheng, C., Liu, S., Xiao, D., Hollembaek, J., Yao, L., Lin, J., Hansel. S. 2010. LC-MS/MS method development and validation for the determination of polymyxins and vancomycin in rat plasma. *Journal of Chromatography B* 878, 2831-2838.

Cipolat-Gotet, C., Cecchinato, A., De Marchi, M., Penasa, M., Bittante, G. 2012. Comparison between mechanical and near-infrared methods for assessing coagulation properties of bovine milk. *Journal of Dairy Science* 95, 6806-6819.

Commission Regulation. 2006a. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Date accessed: 14.04.2013. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:070:0012:0034:EN:PDF

Commission Regulation. 2006b. Commission Regulation (EC) No 1883/2006 of 19 December 2006 laying down methods of sampling and analysis for the official control of levels of dioxins and dioxin-like PCBs in certain foodstuffs. Date accessed: 14.04.2013. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0032:0043:EN:PDF

Commission Regulation. 2006c. Commission Regulation (EC) No 1882/2006 of 19 December 2006 laying down methods of sampling and analysis for the official control of the levels of nitrates in certain foodstuffs. Date accessed: 14.04.2013. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32006R1882:en:NOT

Commission Regulation. 2007. Commission Regulation (EC) No 333/2007 of 28 March 2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs. Date accessed: 14.04.2013. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:088:0029:0038:EN:PDF

Cottyn, B.G., Boucque, C.V. 1968. Rapid method for the gas-chromatographic determination of volatile fatty acids in rumen fluid. *Journal of Agricultural and Food Chemistry* 16, 105-107.

de Rijke, E., Out, P., Niessen, W.M.A., Ariese, F., Gooijer, C., Brinkman, U.A.T. 2006 Analytical separation and detection methods for flavonoids. *Journal of Chromatography A* 1112, 31–63.

DeFrain, J.M., Hippen, A.R., Kalscheur, K.F., Jardon, P.W. 2004. Feeding glycerol to transition dairy cows: effects on blood metabolites and lactation performance. *Journal of Dairy Science* 87, 4195-4206.

Dettmer, K., Aronov, P.A., Hammock, B.D. 2007. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews* 26, 51-78.

Dijkstra, J., Boer, H., Van Bruchem, J., Bruining, M., Tamminga, S. 1993. Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. *British Journal of Nutrition* 69, 385-396.

Eurostat. 2012. Agricultural products. European Commission. Date accessed: 14.04.2013. http://epp.eurostat.ec.europa.eu/statistics_explained/index.php/Agricultural_products

Faulkner, A., Peaker, M. 1982. Reviews of the progress of dairy science: secretion of citrate into milk. *Journal of Dairy Research* 49, 159-169.

Frederiksen, P.D., Hammershøj, M., Bakman, M., Andersen, P.N., Andersen, J.B., Qvist, K.B., Larsen, L.B. 2011. Variations in coagulation properties of cheese milk from three Danish dairy breeds as determined by a new free oscillation rheometry-based method. *Dairy Science & Technology* 91, 309-321.

Furusawa, N. 1999. Rapid liquid chromatographic determination of oxytetracycline in milk. *Journal of Chromatography A*, 839, 247–251.

Garnsworthy, P.C., Masson, L.L., Lock, A.L., Mottram, T.T. 2006. Variation of Milk Citrate with Stage of Lactation and De Novo Fatty Acid Synthesis in Dairy Cows. *Journal of Dairy Science* 89, 1604-1612.

Gentili, A. 2007. LC-MS methods for analyzing anti-inflammatory drugs in animal-food products. *Trends in Analytical Chemistry* 26, 595-608.

Gentili, A., Perret, D., Marchese, S. 2005. Liquid chromatography-tandem mass spectrometry for performing confirmatory analysis of veterinary drugs in animal-food products. *TrAC Trends in Analytical Chemistry* 24, 704-733.

Gopal, P.K., Gill, H.S. 2000. Oligosaccharides and glycoconjugates in bovine milk and colostrum. *British Journal of Nutrition* 84 Suppl 1, S69-74.

Grandison, A.S., Ford, G.D., Owen, A.J., Millard, D. 1984. Chemical composition and coagulating properties of renneted Friesian milk during the transition from winter rations to spring grazing. *Journal of Dairy Research* 51, 69-78.

Grimley, H., Grandison, A., Lewis, M. 2009. Changes in milk composition and processing properties during the spring flush period. *Dairy Science & Technology* 89, 405-416.

Harmeyer, J. 2002. The physiological role of L-Carnitine, Lohmann Information, 27, 1-8. http://lohmann-information.com/content/l_i_27_article_3.pdf

Hop, C.E.C.A, Bakhtiar, R. 1997. An introduction to electrospray ionization and matrix-assisted laser desorption/ionization mass spectrometry: Essential tools in a modern biotechnology environment. *Biospectroscopy* 3 (4), 259-280.

Huntington, G.B. 1997. Starch utilization by ruminants: from basics to the bunk. *Journal of Animal Science* 75, 852-867.

Ikonen, T., Ahlfors, K., Kempe, R., Ojala, M., Ruottinen, O. 1999. Genetic parameters for the milk coagulation properties and prevalence of noncoagulating milk in Finnish dairy cows. *Journal of Dairy Science* 82, 205-214.

Jõudu, I. 2008. Effect of milk protein composition and genetic polymorphism on milk rennet coagulation properties. PhD thesis, Estonian University of Life Sciences. https://dspace.emu.ee/bitstream/handle/10492/113/Thesis_Joudu_2008.pdf?sequence=1

Jõudu, I., Henno, M., Kaart, T., Püssa, T., Kärt, O. 2008. The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows. *International Dairy Journal* 18, 964-967.

Jõudu, I., Henno, M., Värv, S., Viinalass, H., Püssa, T., Kaart, T., Arney, D., Kärt, O. 2009. The effect of milk proteins on milk coagulation properties in Estonian dairy breeds. *Veterinarija Ir Zootechnika* 46, 14-19.

Kass, M., Ariko, T., Kaart, T., Rihma, E., Ots, M., Arney, D. Kärt, O. 2012. Effect of replacement of barley meal with crude glycerol on lactation performance of primiparous dairy cows fed a grass silage-based diet. *Livestock Science* 150, 240-247.

Kim, C.H., Choung, J.J., Chamberlain, D.G. 2001. Estimates of the efficiency of transfer of L-histidine from blood to milk when it is the first-limiting amino acid for secretion of milk protein in the dairy cow. *Journal of the Science of Food and Agriculture* 81, 1150-1155.

Kind, T., Fiehn, O. 2007. Seven Golden Rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. *BMC Bioinformatics* 8, 105-125.

Klandar, A.H., Lagaude, A., Chevalier-Lucia, D. 2007. Assessment of the rennet coagulation of skim milk: A comparison of methods. *International Dairy Journal* 17, 1151-1160.

Klein, M.S., Almstetter, M.F., Schlamberger, G., Nürnberger, N., Dettmer, K., Oefner, P.J., Meyer, H.H.D., Wiedemann, S., Gronwald, W. 2010. Nuclear magnetic resonance and mass spectrometry-based milk metabolomics in dairy cows during early and late lactation. *Journal of Dairy Science* 93, 1539-1550.

Klein, M.S., Buttchereit, N., Miemczyk, S.P., Immervoll, A.K., Louis, C., Wiedemann, S., Junge, W., Thaller, G., Oefner, P.J., Gronwald, W. 2012. NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. *Journal of Proteome Research* 11, 1373-1381.

Krishnan, A., Williams, L.J., McIntosh, A.R., Abdi, H. 2011. Partial Least Squares (PLS) methods for neuroimaging: a tutorial and review. *Neuroimage* 56, 455-475.

Kübarsepp, I., Henno, M., Kärt, O., Tupasela, T. 2005a. A comparison of the methods for determination of the rennet coagulation properties of milk. *Acta Agriculturae Scandinavica Section A - Animal Science* 55, 145-148.

Kübarsepp, I., Henno, M., Viinalass, H., Sabre, D. 2005b. Effect of κ-casein and ß-lactoglobulin genotypes on the milk rennet coagulation properties. *Agronomy Research* 3, 55–64.

Larsen, M., Lund, P., Weisbjerg, M.R., Hvelplund, T. 2009. Digestion site of starch from cereals and legumes in lactating dairy cows. *Animal Feed Science and Technology* 153, 236-248.

Linzell, J.L., Mepham, T.B., Peaker, M. 1976. The secretion of citrate into milk. *Journal of Physiology* 260, 739-750.

Lucey, J.A. 2002. CHEESE | Rennet Coagulation of Milk. In H. Roginski (Ed.), Encyclopedia of Dairy Sciences, 286-293. Oxford, UK, Elsevier.

Mach, N., Bach, A., Devant, M. 2009. Effects of crude glycerin supplementation on performance and meat quality of Holstein bulls fed high-concentrate diets. *Journal of Animal Science* 87, 632-638.

Mortensen, G.K., Main, K.M., Andersson, A., Leffers, H., Skakkebæk, N.E. 2005. Determination of phthalate monoesters in human milk, consumer milk, and infant formula by tandem mass spectrometry (LC–MS–MS). *Analytical and Bioanalytical Chemistry* 382, 1084-1092.

Murphy, M.R., Baldwin, R.L., Koong, L.J. 1982. Estimation of Stoichiometric Parameters for Rumen Fermentation of Roughage and Concentrate Diets. *Journal of Animal Science* 55, 411-421.

O'Mahony, J., Clarke, L., Whelan, M., O'Kennedy, R., Lehotay, S.J., Danaher, M. 2013. The use of ultra-high pressure liquid chromatography with tandem mass spectrometric detection in the analysis of agrochemical residues and mycotoxins in food - Challenges and applications. *Journal of Chromatography A* 1292, 83-95.

Ogola, H., Shitandi, A., Nanua, J. 2007. Effect of mastitis on raw milk compositional quality. *Journal of Veterinary Science* 8, 237-242.

Olsen, R.L. 1989. Effects of Polysaccharides on Rennet Coagulation of Skim Milk Proteins. *Journal of Dairy Science* 72, 1695-1700.

Ørskov, E.R. 1986. Starch digestion and utilization in ruminants. *Journal of Animal Science* 63, 1624-1633.

Ostersen, S., Foldager, J., Hermansen, J.E. 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. *Journal of Dairy Research* 64, 207-219.

Pathak, A.K. 2008. Various factors affecting microbial protein synthesis in the rumen. *Veterinary World* 1, 186-189.

Pires, M., Gatti, C.A., Orellana, G.A., Morales, E. 2004. Rennet coagulation of casein micelles and heated casein micelles in presence of sucrose or lactose. *Food Research International* 37, 95-101.

Politis, I., Ng-Kwai-Hang, K.F. 1988 Effects of Somatic Cell Count and Milk Composition on Cheese Composition and Cheese Making Efficiency. *Journal of Dairy Science* 71, 1711-1719.

Politis, I., Ng-Kwai-Hang, K.F., Giroux, R.N. 1989. Environmental factors affecting plasmin activity in milk. *Journal of Dairy Science* 72, 1713-1718.

Pretto, D., Kaart, T., Vallas, M., Jõudu, I., Henno, M., Ancilotto, L., Cassandro, M., Pärna, E. 2011. Relationships between milk coagulation property traits analyzed with different methodologies. *Journal of Dairy Science* 94, 4336-4346.

Ragaller, V., Lebzien, P., Südekum, K.H., Hüther, L., Flachowsky, G. 2011. Pantothenic acid in ruminant nutrition: a review. *Journal of Animal Physiology and Animal Nutrition* 95, 6-16.

Ruhaak, L.R., Lebrilla, C.B. 2012. Analysis and role of oligosaccharides in milk. *BMB Reports* 45, 442-451.

Salaün, F., Mietton, B., Gaucheron, F. 2005. Buffering capacity of dairy products. *International Dairy Journal* 15, 95-109.

Scalbert, A., Brennan, L., Fiehn, O., Hankemeier, T., Kristal, B.S., van Ommen, B., Pujos-Guillot, E., Verheij, E., Wishart, D., Wopereis, S. 2009. Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. *Metabolomics* 5, 435-458.

Schaar, J. 1984. Effects of kappa-casein genetic-variants and lactation number on the renneting properties of individual milks. *Journal of Dairy Research* 51, 397-406.

Sharif, A., Muhammad, G. 2008. Somatic Cell Count as an Indicator of Udder Health Status Under Modern Dairy Production: a Review. *Pakistan Veterinary Journal* 28, 194-200.

Sørensen, L.K., Elbæk, T.H. 2005. Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. *Journal of Chromatography B* 820, 183-196.

Steijns, J.M., van Hooijdonk, A.C.M. 2000. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *British Journal of Nutrition* 84, 11-17.

Summer, A., Formaggioni, P., Malacarne, M., Sandri, S., Mariani, P. 2003. Composition, acidity and rennet-coagulation properties of early- and late-lactation milks from Italian Friesian cows. *Veterinary Research Communications* 27 Suppl 1, 269-272.

Sundekilde, U.K., Barile, D., Meyrand, M., Poulsen, N.A., Larsen, L.B., Lebrilla, C.B., German, J.B., Bertram, H.C. 2012. Natural variability in bovine milk oligosaccharides from Danish Jersey and Holstein-Friesian breeds. *Journal of Agricultural and Food Chemistry* 60, 6188-6196.

Sundekilde, U.K., Frederiksen, P.D., Clausen, M.R., Larsen, L.B., Bertram, H.C. 2011. Relationship between the metabolite profile and technological properties of bovine milk from two dairy breeds elucidated by NMR-based metabolomics. *Journal of Agricultural and Food Chemistry* 59, 7360-7367.

Tao, N., DePeters, E.J., Freeman, S., German, J.B., Grimm, R., Lebrilla, C.B. 2008. Bovine milk glycome. *Journal of Dairy Science* 91, 3768-3778.

Tao, N., DePeters, E.J., German, J.B., Grimm, R., Lebrilla, C.B. 2009. Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. *Journal of Dairy Science* 92, 2991–3001.

Tiemeyer, W., Stohrer, M., Giesecke, D. 1984. Metabolites of nucleic acids in bovine milk. *Journal of Dairy Science* 67, 723-728.

Tyrisevä, A.M., Elo, K., Kuusipuro, A., Vilva, V., Janonen, I., Karjalainen, H., Ikonen, T., Ojala, M. 2008. Chromosomal regions underlying noncoagulation of milk in Finnish Ayrshire cows. *Genetics* 180, 1211-1220.

Tyrisevä, A.M., Ikonen, T., Ojala, M. 2003. Repeatability estimates for milk coagulation traits and non-coagulation of milk in Finnish Ayrshire cows. *Journal of Dairy Research* 70, 91-98.

Tyrisevä, A.M., Vahlsten, T., Ruottinen, O., Ojala, M. 2004. Noncoagulation of milk in Finnish Ayrshire and Holstein-Friesian cows and effect of herds on milk coagulation ability. *Journal of Dairy Science* 87, 3958-3966.

Urashima, T., Saito, T., Nakamura, T., Messer, M. 2001. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate Journal* 18, 357-371.

Vabariiklik Söötmisalase Uurimistöö Koordineerimise Komisjon. 1995. Ü. Oll, S. Tölp (Toim.), Põllumajandusloomade söötmisnormid koos söötade tabelitega. Tartu, Eesti Põllumajandusülikool (In Estonian)

Vallas, M., Bovenhuis, H., Kaart, T., Pärna, K., Kiiman, H., Pärna, E. 2010. Genetic parameters for milk coagulation properties in Estonian Holstein cows. *Journal of Dairy Science* 93, 3789-3796.

Visser, J., Schaier, R.W., van Gorkom, M. 1979. The role of calcium, phosphate and citrate ions in the stabilization of casein micelles. *Journal of Dairy Research* 46, 333-335.

Waldo, D.R. 1973. Extent and Partition of Cereal Grain Starch Digestion in Ruminants. *Journal of Animal Science* 37, 1062-1074.

Walstra, P. 1984. Variability. In P. Walstra, R. Jenness, H.T. Badings (Eds.), Dairy Chemistry and Physics. 146-161. New York, USA, A Wiley-Interscience Publications.

Wishart, D.S. 2008. Metabolomics: applications to food science and nutrition research. *Trends in Food Science & Technology* 19, 482-493.

SUMMARY IN ESTONIAN

Lehmapiima metaboolse profiili ja laapumise vahelised seosed, söötmise ja laktatsiooniperioodi mõju

Sissejuhatus

Peamine Euroopas toodetav piimatoode on juust: 2011. aastal toodetud 140 miljonist tonnist piimast kulus 36% just juustu tootmiseks (Eurostat, 2012) ning enamik juustudest toodeti ensümaatilise laapumise teel.

Piima laapumisomadustel on suur majanduslik tähtsus, sest see mõjutab nii juustu saagist kui ka kvaliteeti (Bittante *et al.*, 2012). Juustutootmise efektiivsus sõltub piimakomponentide kadudest vadakus, mistõttu on tähtsad nii piima laapuvuse ajaline ühtlus kui ka kalgendi tugevus, sest nende kaudu on võimalik vähendada kadusid (Cecchinato *et al.*, 2013). Kalgendi moodustumiseks soovitud aega on võimalik optimeerida laapensüümi kontsentratsiooni suurendamisega, kuid samas võib see kahjulikult mõjuda juustu saagikusele ja suurendada mõrude peptiidide osakaalu lõpptootes (Cecchinato *et al.*, 2013).

Lehmapiima laapumine on laialdaselt uuritud pärilik omadus (Ikonen *et al.*, 1999; Cassandro *et al.*, 2008; Vallas *et al.*, 2010; Bittante *et al.*, 2012), mis peale geenide on mõjutatud veel laktatsioonijärgust, tõust ja söötmisest (Jõudu, 2008; artikkel **I**). Kahjuks pole tänaseks veel teada kindlaid geneetilisi või metaboolseid tegureid, mis lubaks piima laapumisomadusi seletada ja prognoosida. Lisaks geneetilistele uuringutele on alustatud ka metaboloomika-alaste uuringutega molekulaarsel tasemel, saamaks teada, kuidas parendada toorpiima laapumisomadusi ja seeläbi suurendada juustutootmise efektiivust.

Metaboloomika on teadus, mis tegeleb organismis leiduvate madalmolekulaarsete molekulide (< 1500 Da) määramise ja identifitseerimisega (Wishart, 2008). Ajalooliselt alustati metaboliitide analüüsimist tuumamagnetresonants-spektromeetriaga (NMR), kuid tänapäeval kasutatakse kõrge tundlikkuse ja suure lahutuvusvõime tõttu pigem mass-spektromeetriat (MS) (Dettmer *et al.*, 2007). Piima metaboliitide uurimisel on enamasti piirdutud eesmärgiga tagada pestitsiidide, mükotoksiinide ja antibiootikumide ning nende metaboliitide vaba tervislik toit (Gentili *et al.*, 2005; Sørensen, Elbæk, 2005; Blasco

et al., 2009). Seni on publitseeritud ainult kaks artiklit, mis kirjeldavad lehmapiima tehnoloogilisi omadusi piimale omaste metaboliitide kaudu (Sundekilde *et al.*, 2011; artikkel **I**).

Antud uurimustöö kirjeldab esimest laialdast lehmapiima metaboolse profiili määramist Eestis. Doktoritöö käigus uuriti erinevate laapumisomadustega lehmapiimade metaboolset profiili ning selle seotust söötmise ja laktatsioonistaadiumiga.

Töö hüpoteesid on:

1. Piima molekulaarse koostise põhjal on võimalik hinnata piima laapumisomadusi.
2. Piima laapumisomadused ja nende seos metaboolse profiiliga on mõjutatud laktatsioonistaadiumist ja söötmisest.

Lähtuvalt hüpoteesidest on töö eesmärkideks:

1. Töötada välja kiire ja robustne piima eeltöötlemise juhis, mis võimaldaks määrata madalmolekulaarseid ühendeid vedelikkromatograaf-tandem-mass-spektromeetril (LC-MS/MS) (**I, II**). Antud metoodika võimaldaks leida spetsiifilisi laapuvust iseloomustavaid metaboolseid markereid, mis omakorda täiendaks teadmisi molekulaarsel tasandil toimuvatest laapumisega seonduvatest protsessidest.
2. Saada ülevaade erinevate laapumisomadustega piimade metaboolsetest profiilidest. Leida spetsiifilised laapumisega seostuvad markerid, analüüsides ekstreemsete laapumisomadustega (mittelaapuvad ja väga hästi laapuvad) piimasid (**I**).
3. Analüüsides toorglütserooli söönud lehmade piima, leida söötmise mõju piima laapumisomadustele ja metaboolsele profiilile (**III**).
4. Määrata, millises ulatuses muutuvad piima metaboolne profiil ja laapumine laktatsiooni käigus (**IV**).

Metoodika

Antud uurimustöö on kokkuvõte neljast erinevast katsest, mille ülesehitus on toodud tabelis 1.

Piima koostis määrati Jõudluskontrolli Keskuses automaatse piimaanalüsaatoriga (CombiFoss 6000, Foss Electric, Hillerød, Denmark) ning laapumisnäitajad optigraafil (Alliance Instruments, Frepillon, France)

Eesti Maaülikooli veterinaarmeditsiini ja loomakasvatuse instituudis piima kvaliteedi uurimise laboratooriumis Kübarsepp *et al.* (2005) metoodika järgi. Laapumise kirjeldamiseks määrati RCT (laapumiseks kulunud aeg ehk minutid, mis kulusid laapensüümi lisamisest kuni kalgendi moodustumise alguseni) ja E_{30} (kalgendi tugevus millimeetrites 30 minutit pärast laapensüümi lisamist). Vastavalt kalgendi tugevusele jaotati piimad mittelaapuvateks ($E_{30} = 0$ mm), halvasti ($0 < E_{30} < 20$ mm) ja hästi ($E_{30} \geq 20$ mm) laapuvateks piimadeks (Tyrisevä *et al.*, 2004).

Tabel 1. Katsete ülesehitus.

Artikli nr	Piimaproovid	Proovide arv	Piima-analüüsid	Lisa-analüüsid
I	Üks kord kuus jõudluskontrolli käigus kogutud üksiklehmade piimad. Rõhk mittelaapuvatel ja hästi laapuvatel piimadel.	143	Piima koostis, pH, RCT[1], E_{30} [2], MS-analüüsid	
II	Laktatsiooni esimese saja päeva jooksul kaks korda nädalas kogutud üksiklehmade piimad	133	Piima koostis, pH, MS-analüüsid	Kogutud vereproovide (n = 133) MS-analüüsid[3]
III	Kaheksa primipaarse lehma piimad. 4x4 ladina ruudu katse, kus lehmade ratsioonis üks, kaks või kolm kg odrajahu asendati vastava koguse glütserooliga	64	Piima koostis, pH, RCT, E_{30}, MS-analüüsid	Vereplasma insuliini- ja glükoosi-sisaldus[3], nelja lehma vatsavedeliku lenduvate rasvhapete sisaldus
IV	Ühe aasta vältel üks kord kuus jõudluskontrolli käigus kogutud üksiklehmade piimad.	306	Piima koostis, pH, RCT, E_{30}, MS-analüüsid	

[1] RCT - laapumiseks kulunud aeg (min), mis kulus laapensüümi lisamisest kalgendi moodustumise alguseni; [2] E_{30}- kalgendi tugevus (mm) 30 minutit pärast laapensüümi lisamist; [3] ei arutata antud uurimustöös.

Kogutud piimaproovid säilitati −20 °C juures ning enne analüüsimist sulatati vesivannis (40 °C). Analüüsitavast proovist rasva eemaldamiseks piim tsentrifuugiti (3000 x g, 10 min, 4 °C; Refrigerated Centrifuge 2-16K, Sigma-Aldrich, St. Louis, USA). Valkude sadestamiseks lisati rasvavabale piimale võrdses koguses 3%list trikloroäädikhappe lahust ning tsentrifuugiti (17 700 x g, 15 min, 4 °C). Eraldunud pealmisele vedelikukihile lisati samas koguses atseetonitriili ja tsentrifuugiti (15 644 x g, 15 min, 4 °C; Hettich Zentrifugen Universal 32R, GMI Inc., Ramsey, USA). Saadud lahust analüüsiti edasi kolmekordsel kvadrupool-lineaarsel ioonlõks-mass-spektromeetril (LC-MS/MS; 3200 Q TRAP; AB Sciex Instruments, Foster City, USA). Positiivne ja negatiivne ioniseerimine saavutati kasutades Turbo Ion Spray'd. Metaboolne profiil määrati toatemperatuuril (EMS; **I, II, III, IV**) ning spetsiifilised ühendid 200 °C juures (MRM; **I, II, III)**. Erinevate ühendite määramiseks kasutati C_{18} (Luna 3 µm C18 100A, 100 x 2.00 mm, Phenomenox, Torrance, USA) ja HILIC (Luna 5µm HILIC 200 A, 150 x 3.00 mm, Phenomenox, Torrance, USA) kolonne (**I, II, III)**. Markerite identifitseerimiseks (**I, II, III, IV**) nende massi ja laengu suhte (*m/z*) alusel kasutati erinevaid andmebaase nagu MassBank (http://www.massbank.jp/), the Human Metabolome Database (HMDB; http://www.hmdb.ca/) ja METLIN Metabolome Database (http://metlin.scripps.edu/). Statistiliselt töödeldi andmed statistikaprogrammidega SAS (version 9.1; SAS Institute Inc., Cary, NC, USA) ja R 2.8.1 Bioconductor.

Tulemused

Antud uurimustöö käigus välja töötatud piima eeltöötlemise ja määramismetoodika on olnud sobiv leidmaks madalmolekulaarseid ühendeid LC-MS/MS-il. Metaboolsete profiilide erinevused mittelaapuvate ja hästi laapuvate piimade vahel on visuaalselt eristatavad joonisel 2.

MS-analüüsiga määratud ühendite korreleerumist somaatiliste rakkude arvuga (SRA) uuriti Pearsoni korrelatsioonanalüüsi kasutades ning täheldati, et korrelatsioon metaboolse profiili ja SRA vahel pole tugev. SRA mõju uuriti ka peakomponent-analüüsiga (PCA; joonis 3). Tulemused näitavad, et mittelaapuvad ja hästi laapuvad piimad on erinevad, olenemata somaatiliste rakkude sisaldusest. Seega molekulaarsel tasemel toimuvaid laapuvust kirjeldavaid protsesse SRA ei mõjuta.

Statistiline olulisus laapuvate ja mittelaapuvate piimade *m/z* väärtuste intensiivsuste vahel leiti kasutades Student t-testi ja määrati p-väärtus. Saadud olulisus visualiseeriti *heat-map*-iga (joonis 4), näidates signaalide intensiivsuste suhet keskmisesse vastava *m/z* väärtuse korral. *M/z* väärtused järjestati arvutatud statistiliste erinevuste järgi. Metoodika võimaldas leida laapuvusega seostatavaid signaale nagu *m/z* = 162. Antud signaal peaks vastama L-karnitiini spektrile. Teistele ainetele kindlaid vasteid ei leitud, kuigi paljud spektrid olid sarnased. L-karnitiin ehk B_T-vitamiin on nii lihase, vere kui ka piima koostisosa ja seda peetakse vajalikuks bioaktiivseks ühendiks, mis osaleb rasvhapete (> C_{14}) transpordil mitokondrisse ja lipolüüsi inhibeerimises. L-karnitiini sisaldus piimas sõltub nii laktatsiooniperioodist, laktatsiooninumbrist kui ka piimatoodangust, langedes laktatsiooni edenedes, lehma vanuse tõustes ja kõrgema toodangu korral (Harmeyer, 2002).

Läbi viidud fragmentanalüüsi käigus täheldati erinevate ühendite vahel sarnasusi: (1) erinevate ühendite fragmendid olid sarnased (*m/z* 99, 180 ja 261), (2) suurema massiga ühendid sisaldasid väiksema massiga ühendeid kui fragmente, (3) sarnane oli ka fragmentide vähenemine *m/z* 18 kaupa. Edasine analüüs näitas, et võrdlusaine N-atestüüllaktosamiini (*m/z* = 365) fragmenteerimisspekter vastas meile tundmatule signaalile *m/z* 365. Mittelaapuvates piimades oli viiest võimalikust signaalist neljas see signaal olemas, hästi laapuvas piimas sellist markerit ei leitud. Kuna erinevad oligosahhariidid annavad samu spektreid, tuleb saadud tulemustesse suhtuda ettevaatlikult ning jätkata uuringuid, kuidas ja kas oligosahhariidid laapumist mõjutavad (**I**).

Piima laapumisomadusi ja metaboolseid profiile analüüsiti ka toorglütserooli söönud lehmade piimades (**III**). Katse eesmärk oli uurida, kas ja kuidas glükogeense prekursori (odrajahu *vs* glütserool) vahetus söötmisel mõjutab piima laapumisomadusi. Söötmiskatse käigus asendati lehmade ratsioonis üks, kaks või kolm kg odrajahu vastava koguse glütserooliga. Katse käigus piima üldine koostis erinevate söötmisgruppide vahel oluliselt ei erinenud, v.a piima valgusisaldus, mis viitab suurenenud mikrobiaalse proteiini sünteesile vatsas (Pathak, 2008). Suurenenud proteiinikogus oli positiivses seoses piimakalgendi tugevusega ($r = 0{,}58$, $P < 0{,}001$).

Antud katse käigus leiti, et glütserooli söötmine parandab piima laapuvust ning teatud metaboliidid on omased heale laapuvusele (joonis 5). Piima väga head laapuvust iseloomustas positiivselt laetud signaal m/z 293, mis erinevate andmebaaside kohaselt omas nii histidiini- kui ka lüsiinimolekulidele omaseid piike. Kirjanduse põhjal (Kim *et al.*, 2001) on histidiin, lüsiin ja metioniin piimavalgu sünteesi limiteerivateks aminohapeteks. Seega mõlema aminohappe koguste suurenemisel peaks suurenema ka piima valgusisaldus. Antud katse käigus oli glütserooli osakaalu tõstmisel lehmade söödaratsioonis piima valgusisalduse suurenemine märgatav ($P < 0,05$).

Peale eelnevalt mainitud positiivse signaali (m/z 293) oli viis statistiliselt olulist ($P < 0,05$) negatiivset signaali (m/z 218, 292, 333, 421 ja 422), mis seostusid hea laapumise ning suurema glütserooli koguse söötmisega. Signaali m/z 292 fragmentspekter vastas glütserofosfokoliini spektrile. Klein *et al.* (2012) leidis, et glütserofosfokoliini ja fosfokoliini suhe piimas on hea ketoosimarker ehk suurenenud glütserofosfokoliini tase viitab väiksemale ketoosiriskile. Signaali m/z 218 fragmentanalüüs andis pantoteenhappele omaseid fragmente. Mäletsejad saavad vajaliku pantoteenhappe nii söödast kui ka vatsa mikroorganismide sünteesi tulemusel (Ragaller *et al.*, 2011). Vaba pantoteenhappe olemasolu piimas on haruldane, tavaliselt on see seotud koensüüm A-ga, osaledes raku energia ainevahetusel (Ragaller *et al.*, 2011).

Piimas leiduv tsitraat võib läbi teiste ühendite omada mõju piima tehnoloogilistele omadustele (nt laapuvusele), parandades kaltsiumi- ja fosfori-ioonide sidumist kaseiinimitselliga (Garnsworthy *et al.*, 2006; Visser *et al.*, 1979). Nimi juba viitab, et tsitraat on tähtis tsitraaditsükli vaheühend, võttes osa raku energia ainevahetusest. Seetõttu peetakse tsitraati lehma energeetilise taseme indikaatoriks (Baticz *et al.*, 2002). Tsitraat ei suuda läbida udara näärmekoe epiteeli (Linzell *et al.*, 1976), mistõttu see toodetakse udara näärmekoes. On leitud, et selle kontsentratsioon on mõjutatud nii laktatsiooni faasist (Garnsworthy *et al.*, 2006) kui ka söötmisest (Faulkner, Peaker, 1982). Samas Gransworthy *et al.* (2006) väidab, et tsitraadi kontsentratsioon ei sõltu piima kogusest, söödast ega mastiidist, kuid selle kontsentratsioon on kõrgem laktatsiooni alguses ning seostatav rasvhapete oksüdatsiooniga. Kirjandusallikate vastuolulise info tõttu mõõdeti erinevate söödaratsioonide jooksul kogutud piimaproovide tsitraaditsükli erinevate ühendite sisaldust (tabel 2

artiklis **III**). Ainult malonaat ja oksaloatsetaat olid positiivselt seostatavad kalgendi tugevusega (r = 0,25, *P* = 0,06 ja r = 0,33, *P* = 0,013), ülejäänud tsitraaditsükli komponentide sisaldus piimaproovides katse käigus oluliselt ei muutunud (joonis 6). Siit tulenevalt lehmade söödaratsioonis odrajahu osaline asendamine toorglütserooliga ei omanud tsitraadi kontsentratsioonile mingit mõju.

Kui vasikas kasvab, muutub tema toitainete vajadus ning vastavalt peab muutuma ka lehmapiima koostis (Walstra, 1999). Võib-olla just tänu antud füsioloogilisele eripärale on laapumist (Ostersen *et al.*, 1997; Summer *et al.*, 2003; Tyrisevä *et al.*, 2003) ja selle muutumist laktatsiooni ajal (Vallas *et al.*, 2010) nii palju uuritud. Piim laapub kõige paremini laktatsiooni alguses, halvemini keskel ja laktatsiooni lõpus jälle paraneb (Ostersen *et al.*, 1997; Summer *et al.*, 2003; Tyrisevä *et al.*, 2003). Seepärast uuriti ka antud töö käigus piima metaboolset profiili laktatsiooni jooksul ja selle seost laapuvusega. Laktatsioon jaotati kolmeks perioodiks: esimesed 60 päeva, 61 kuni 240 päeva ja viimased 60 päeva.

Uuritud proovide kalgendi tugevus oli laktatsiooni vältel ühtlaselt hea (joonis 7; tabel 1 artiklis **IV**) ning saadud tulemused ühtisid varasemate uuringutega (Ostersen, *et al.*, 1997). Laapumiseks kulunud aeg oli sarnane Vallas *et al.* (2010) ja Ostersen *et al.* (1997) avaldatud tulemustega, olles lühim laktatsiooni alguses (alla 6 min). Eelnevalt (Grandison *et al.*, 1984; Ostersen *et al.*, 1997; Kübarsepp *et al.*, 2005a) on RCT suurenemist seostatud pH tõusmisega. Lisaks leiti, et piima laapumisomadused on tugevalt lehmaspetsiifilised (tabel 1 artiklis **IV**) ja mõjutatud lüpsiajast: tugevam kalgend ja lühem laapumisaeg oli õhtuse lüpsi piimadel.

Kuigi muutused piima metaboloomi ja laapumise vahel laktatsiooni erinevatel perioodidel ei korreleerunud tugevalt, olid need siiski enam-vähem samasuunalised ja sarnased kõigi kolme perioodi vältel (joonis 6 artiklis **IV**). Ainult kaks positiivselt (m/z = 197 ja 342) ja kaheksa negatiivselt (m/z = 612, 737, 835, 836, 902, 1000, 1038 ja 1079) ioniseeritud ühendit korreleerusid kalgendi tugevusega positiivselt (r > 0,3). Laapumiseks kulunud ajaga ei seostunud ühtegi signaali. Seega on laktatsiooni erinevatel perioodidel esinevaid muutusi kalgendi tugevuses võimalik seostada muutustega teatud metaboliitides, kuid sama ei saa väita laapumiseks kuluva aja kohta.

Järeldused

- Saadud tulemused kinnitavad, et välja töötatud metoodika piima eeltöötlemiseks (rasva ja valgu eemaldamine) sobib mass-spektromeetriliseks (LC-MS/MS) madalmolekulaarsete ühendite määramiseks ja metaboolse profiili leidmiseks. Teatud signaale (nt L-karnitiin) saab seostada laapuvuse või teiste piima iseloomustavate tunnustega, mis võivad mõjutada laapuvust (nt SRA). Samuti saaks neid seostada teiste organismis toimuvate protsessidega (**I, II**).

- Erinevate tehnoloogiliste omadustega (nt hea ja halb laapuvus) piimade metaboloomi erinevust on võimalik määrata LC-MS/MS-analüüsil. Senini pole suudetud detailselt kirjeldada, mil määral antud uuringu käigus juba määratud metaboliidid (L-karnitiin ja pantoteenhape) parendavad või halvendavad laapuvust, kuid esitatud tulemused on siiski samm selles suunas (**I, III, IV**).

- Esialgsed tulemused näitavad, et glükogeense prekursori vahetus lehma söödas mõjutab piima metaboolset profiili. Piima laapumisomadused võivad paraneda seoses lenduvate rasvhapete proportsiooni muutusega vatsas ning olla seotud valgu ja energeetiliste metaboliitide (nt tsitraaditsükli komponendid, pantoteenhape) kontsentratsiooni muutustega piimas. Seega piimalehmadele söödetud toorglütserool, kui alternatiivne glükoneogeneesi allikas, näib omavat soodsat mõju laapuvusele (**III**).

- Katsetulemused näitasid kalgendi tugevusele omaste spetsiifiliste metaboliitide olemasolu piimas kogu laktatsiooni vältel. Muutused metaboloomis ei seostunud laapumiseks kuluva ajaga (**IV**).

Tulevik

Tööd tuleb jätkata laapumisega seotud metaboliitide identifitseerimisega. Hetkel on metaboolne profiil määratud üksiklehmade piimadest, kuid tuleks läbi viia ka tanki- ja juustupiimade metaboolsed analüüsid. Leides täiendavaid juustusaagist kirjeldavaid markereid, mida kasutada sobiva juustupiima valimisel, saame suurendada juustu väljatulekut.

ACKNOWLEDGEMENTS

This research was conducted in the Department of Animal Nutrition and Products Quality at the Estonian University of Life Sciences and the Institute of Bio- and Translational Medicine of the University of Tartu. The study was supported by the Bio-Competence Centre of Healthy Dairy Products LLC, co-financed by the European Community's Regional Development Fund under the framework of the Competence Centre Programme of the Enterprise Estonia project no. EU30002, targeted finance project SF0170165s08, and Estonian Science Foundation grants 7494 and 7856.

My greatest gratitude goes to my supervisors Olav Kärt, Ursel Soomets and Kalle Kilk for all their support, guidance in animal nutrition and in the expanding field of metabolomics, and also for the contribution to the thesis.

I am very thankful to David Arney, Tanel Kaart, Ivi Jõudu and Tiia Ariko for their contributions.

I sincerely thank my co-workers at the Estonian University of Life Sciences and University of Tartu.

I

ORIGINAL PUBLICATIONS

Harzia, H., Kilk, K., Jõudu, I., Henno, M., Kärt, O., Soomets, U. 2012
COMPARISON OF THE METABOLIC PROFILES OF
NONCOAGULATING AND COAGULATING BOVINE MILK
Journal of Dairy Science, 95(2), 533 – 544

J. Dairy Sci. 95:533–540
doi:10.3168/jds.2011-4468

Comparison of the metabolic profiles of noncoagulating and coagulating bovine milk

H. Harzia,*†‡[1] K. Kilk,† I. Jõudu,*‡ M. Henno,* O. Kärt,* and U. Soomets†
*Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Kreutzwaldi 46, Tartu 51006, Estonia
†Department of Biochemistry, Medical Faculty, University of Tartu, the Centre of Excellence for Translational Medicine, Ravila 19, 50411 Tartu, Estonia
‡Bio-Competence Centre of Healthy Dairy Products, Kreutzwaldi 1, 51014 Tartu, Estonia

ABSTRACT

We hypothesize that, through milk composition and different milk metabolites, it is possible to characterize the technological properties (e.g., coagulation) of milk. In this research, liquid chromatography mass spectrometry was used to obtain profiles of low molecular weight organic compounds in 143 milk samples. The metabolic profiles of milk from cows were correlated with their coagulation properties. Using multivariate data analysis methods, we demonstrated that the metabolic profiles of the milk were correlated with coagulation ability. Several marker ions responsible for differential coagulation were found. Although not all affected metabolites could be identified, the most significant differences were found for carnitine and oligosaccharides. Exploitation of these results may increase the use of biomarkers to assess the coagulation ability of milk. This study represents the first large-scale metabolomic profiling of noncoagulating and coagulating bovine milk samples in Estonia.

Key words: milk coagulation, milk metabolite, tandem mass spectroscopy

INTRODUCTION

About 40% of the milk produced in Europe is used to make cheese (Rohner-Thielen, 2008), and most of the cheeses are produced by enzymatic coagulation. Good coagulation properties allow good conversion of milk solids to cheese and profits to the dairy companies. Therefore, the coagulation ability of milk influences cheese yield and quality and thus is very important.

Poor coagulation ability of milk is a problem in Italy (Malossini et al., 1996; Cassandro et al., 2008) and Finland, where about 8.6% of Finnish Ayrshire cows produce noncoagulating milk at least once during a lactation (Tyrisevä et al., 2004). A previous study (Kübarsepp et al., 2005b) in Estonia showed that about 8 to 9% of milk samples did not coagulate, and 17 to 20% of milk coagulated poorly with rennet.

Factors affecting milk coagulation ability have been widely studied. Milk coagulation properties are heritable; up to 40% of the variation is caused by genes (Ikonen et al., 2004; Vallas et al., 2010). The genome-wide scan by Tyrisevä et al. (2008) located the genomic regions associated with the noncoagulation of milk, and 2 potential candidate genes were found (on chromosome 2, *BMS1126* and on chromosome 18, *BMS1355*). Other factors influencing milk coagulation ability include stage of lactation, breed, nutrition, milk pH, SCC, milk composition, and milk protein genotypes (Hooydonk et al., 1986; Ostersen et al., 1997; Tyrisevä et al., 2003; Guinee et al., 2001; Auldist et al., 2002; Ikonen et al., 2004; Wedholm et al., 2006; De Marchi et al., 2007; Jõudu, 2008).

Neither genetics nor the factors described above clearly explain the noncoagulation of milk. It is possible that metabolic methods could be used to identify the most suitable milk for cheese making, based on a small number of easily detectable biomarkers. Metabolomics is the identification of the small molecule metabolites found in an organism. In clinical diagnostics and toxicology, blood or urine is most often used to assess body condition. Milk samples are easier to gather than blood samples, and in precision farming, all analyses regarding animal health could be made at milking. By analyzing milk composition, technological properties, and different milk metabolites, it is possible to evaluate numerous animal health and welfare indicators, as well as the nutritional status of animals. To date, good indicators exist for the early diagnosis of ketosis, but no such reliable, noninvasive methods have been devised for other nutrition-related metabolic diseases (Ingvartsen, 2006).

The aim of this study was to develop a method that would pinpoint the specific metabolic markers describing coagulation ability and provide us with further understanding about the mechanisms involving milk coagulation at the molecular level. If the detected metabolic markers are alterable by nutrition or selec-

Received April 20, 2011.
Accepted October 18, 2011.
[1]Corresponding author: hedi.harzia@emu.ee

tion, this would provide an economic advantage to the dairy industry. This study was a part of a wider study describing bovine milk in Estonia.

MATERIALS AND METHODS

Data Collection and Analysis

Milk samples (n = 143) from healthy cows of 2 different breeds, Estonian Holstein (n = 121) and Estonian Red (n = 22), were collected during the period from September 2008 to May 2010 by using in-line milk meters within the framework of regular animal recording from 39 different farms around Estonia. Milk samples were stabilized with bronopol (Broad Spectrum Microtabs, D&F Control Systems Inc., Norwood, MA). Concentrations of milk fat, protein, SCC, and urea were measured from each milk sample at the Milk Analysis Laboratory of the Estonian Animal Recording Centre (Tartu), using an automated infrared milk analyzer (System 4000, Foss Electric, Hillerød, Denmark). Information on the birth and calving dates of the cows were obtained from the Estonian Animal Recording Centre. Milk pH was measured using a pH meter (MP 220, Mettler Toledo GmbH, Greifensee, Switzerland) before analyzing coagulation ability at 20°C in the Laboratory of Milk Quality at Estonian University of Life Sciences (Tartu).

Coagulation Analysis

For all 143 milk samples, the rennet coagulation properties were determined by using an Optigraph (Alliance Instruments, Frepillon, France) as described by Kübarsepp et al. (2005a) at the Laboratory of Milk Quality at Estonian University of Life Sciences. Two milk coagulation parameters were measured: milk coagulation time (the time taken in minutes to start forming curd from rennet addition) and curd firmness ($\mathbf{E_{30}}$, mm, curd firmness after 30 min of enzyme addition). Milk that did not form a curd was classified as noncoagulating (n = 27, E_{30} = 0 mm) and that with particularly good coagulation ability (n = 27, E_{30} ≥40mm) was classified as well coagulating. The remaining 89 intermediate samples were classified as poor or good, based on their coagulation ability. The Optigraph recorded some curd firmness (E_{30} >0.89 mm) in milk with poor coagulation properties, but following further observation, complete coagulation did not occur. Milk with good coagulation ability did form a firm curd (20 < E_{30} < 40 mm), but such samples were not identified as well-coagulating because the E_{30} value did not reach 40 mm.

Sample Preparation

The milk samples were stored at −20°C until analyzed for metabolites. Frozen samples were thawed in a water bath at temperature 40°C, and a 5-mL aliquot of milk was centrifuged (Refrigerated Centrifuge 2-16K, Sigma-Aldrich, St. Louis, MO) at 3,000 × g for 10 min at 4°C to remove fat. A 3-mL sample of skim milk was mixed with 3 mL of 3% TCA solution (ACS reagent, ≥99.5% purity, Sigma-Aldrich) and centrifuged at 17,000 × g for 15 min at 4°C. The supernatant was mixed with an equal volume of acetonitrile (LC-MS Chromasolv, ≥99.9% purity, Sigma-Aldrich) and centrifuged (Hettich Zentrifugen Universal 32R, GMI Inc., Ramsey, NJ) at 14,000 × g for 15 min at 4°C. The supernatant was collected and injected into the mass spectrometer for further analyses.

Mass Spectrometry

Mass spectrometry analyses were carried out on the triple quadrupole linear ion trap hybrid liquid chromatography-tandem mass spectrometer (**LC-MS/MS**; 3200 Q TRAP; AB Sciex Instruments, Foster City, CA). The sample was injected into the MS using the SIL-20A autosampler (Shimadzu, Kyoto, Japan). The mobile phase (80% acetonitrile in water) was delivered by LC-20AD pumps (Shimadzu). Positive ions formed in the source were scanned in enhanced mode within the m/z mass range 50 to 1,500. The scan rate was 1,000 amu/s. Curtain and nebulizer gas had settings of 10 and 20, respectively. Ionization was performed at room temperature. The ion spray voltage was set at 4,500 V. The entrance and declustering potential and the collision energy were set at 10 V. The system was controlled by the Analyst 1.4.2 software (Applied Biosystems Inc., Foster City, CA).

For preliminary compound identification, C_{18} (Luna 3-μm C18 100A, 100 × 2.00 mm, Phenomenex, Torrance, CA) and hydrophilic interaction liquid chromatography (**HILIC**, Luna 5-μm HILIC 200A, 150 × 3.00 mm, Phenomenex) columns were used. The gradient for retention time studies was as follows: 5 min isocratic at 95% acetonitrile in water, gradient decline to 5% acetonitrile in water within 40 min, 10 min at 5% acetonitrile in water.

Statistical Analysis

To reduce the effects of error, MS spectral data were pre-processed by binning data to atomic mass unit resolution. Statistical analyses of the data were made using in-house R 2.8.1/BioConductor algorithms (R Development Core Team, 2008). Statistical significance between m/z intensities in the spectra of noncoagulating and well-coagulating milk was determined by Student t-tests, and correlations were found using Pearson correlation analyses. Results were assessed with princi-

pal component analysis (**PCA**) and a heat-map that displayed the distribution of signal intensities relative to the mean at respective m/z values. The m/z values were ranked based on calculated statistical differences, and only significant values are presented.

RESULTS AND DISCUSSION

Characteristics of Analyzed Milk Samples

Parallel to the MS-based metabolic profiling study, all milk samples were analyzed for the major milk compounds. Mean values of DIM, SCS [SCS = ln (SCC)], fat, urea, and protein concentrations, pH, and coagulation parameters for all 4 groups of sampled milk are shown in Table 1.

Characteristics for the well-coagulating and noncoagulating groups were compared. Significant differences were found for pH ($P = 9.54 \times 10^{-23}$), SCS ($P = 1.60 \times 10^{-5}$), fat ($P = 8.18 \times 10^{-5}$), urea ($P = 1.69 \times 10^{-7}$), and protein ($P = 1.99 \times 10^{-5}$) contents (Table 1).

According to previous studies (Tyrisevä et al., 2003; Ikonen et al., 2004; Jõudu, 2008) milk coagulation should be optimal at the beginning of lactation, worst during mid-lactation, and improving during late lactation. All of the well-coagulating milk samples were from different stages of lactation; DIM varied from 34 to 320. Noncoagulating milk samples were collected from d 93 to d 338 of lactation and thus did not represent only mid-lactation, as anticipated.

Besides stage of lactation, milk coagulation ability differs at different pH values and SCC levels. In this study, noncoagulating milk had a higher pH (pH = 6.85 to 7.11) than well-coagulating milk (pH = 6.54 to 6.75). This was observed previously (Okigbo et al., 1985; Ikonen et al., 2004). Changes in milk pH can be due to the onset of udder inflammation or stage of lactation (Harmon, 1994; McCarthy and Singh, 2009). All 143 analyzed milk samples were collected from different farms with different feeding practices from cows with no identified diseases during regular animal recording. Therefore, it was not possible to identify the cause of the elevated levels of pH in noncoagulating milk samples in this study.

Somatic cell count can be used as an indicator of udder health, which may affect coagulation ability (Okigbo et al., 1985; O'Brien et al., 2001). In turn, SCC in milk can be affected by stage of lactation, stress, age of the cow, and milking frequency (Kelly, 2003). An elevated level of SCC can occur in both well-coagulating and poorly coagulating milks (Jõudu, 2008), as was the case in our study. To eliminate a possible effect of SCC, a separate database comprising milk samples with an SCC $<5.0 \times 10^5$ cells/mL was prepared that comprised 93 samples, including 8 noncoagulating and 8 well-coagulating samples. The SCC in our 2 new groups (noncoagulating and well-coagulating with low SCC) were still significantly different ($P = 0.003$), as was pH ($P = 1.41 \times 10^{-7}$), fat ($P = 0.013$), and protein ($P = 0.0001$) content. All 8 of the well-coagulating milk samples were from cows in late lactation (238 to 320 DIM), and the same was observed for the noncoagulating milk samples (215 to 284 DIM).

Global Metabolite Profiles

The main hypothesis of this study was that MS analysis of low molecular weight milk compounds could pinpoint metabolites that are correlated with coagulation ability. The validity of this hypothesis, and subsequent identification of the key metabolites, would lead to a better understanding of the mechanisms behind unsatisfactory coagulation. As a first step, MS analyses were performed with no targeting of a specific group of metabolites within the mass range m/z 50 to 1,500 (nontargeted analysis). Illustrative

Table 1. Mean values (SD in parentheses) of DIM, SCS, fat, urea, and protein concentration, pH, and E_{30} for all 143 milk samples

		Intermediate		
Trait[1]	Noncoagulating (n = 27)	Poor (n = 77)	Good (n = 12)	Well-coagulating (n = 27)
DIM	244[a] (55)	182[b] (69)	241[a] (78)	251[ab] (68)
Fat, %	3.82[a] (1.19)	3.86[a] (1.03)	4.24[a] (0.76)	5.05[b] (0.82)
Protein, %	3.71[a] (0.43)	3.58[a] (0.37)	3.76[a] (0.32)	4.19[b] (0.32)
Urea, mg/dL	12.45[a] (6.48)	23.00[b] (9.12)	22.86[b] (6.84)	25.33[b] (7.92)
pH	7.00[a] (0.11)	6.72[b] (0.11)	6.65[c] (0.06)	6.62[c] (0.05)
SCS	6.97[a] (1.22)	5.21[b] (1.92)	5.49[b] (1.23)	5.34[b] (1.29)
E_{30}, mm	0.00[a] (0.00)	20.52[b] (10.56)	28.78[c] (6.61)	47.22[d] (3.65)
RCT, min	21.68[a] (14.05)	16.06[b] (4.46)	9.98[c] (1.46)	8.92[d] (1.10)

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

[1]SCS = ln (SCC); E_{30} = curd firmness after 30 min; RCT = milk coagulation time.

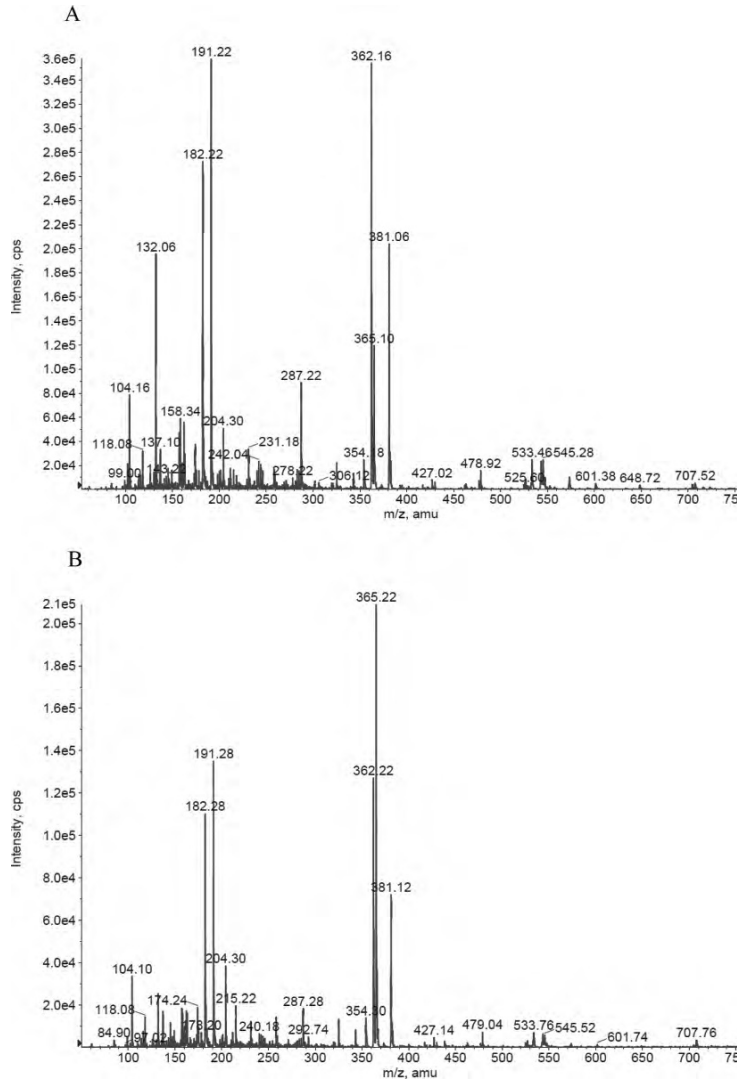


Figure 1. Spectra of (A) noncoagulating milk and (B) well-coagulating milk (m/z 50–750).

spectra of typical examples of noncoagulating and well-coagulating milks are presented in Figures 1A and 1B, respectively.

Although differences between the well-coagulating and noncoagulating milk samples were apparent from individual spectra, PCA and calculations for statisti-

cally significant differences were performed to better identify these differences.

PCA. First, the metabolic spectra of all 143 milk samples (all 4 coagulating groups) were analyzed. The samples with intermediate coagulation ability were distributed around the same area as the well-coagulating and noncoagulating samples without forming a well-defined cluster of their own (Figure 2A). We observed that, among the intermediate group, the better coagulating samples were located closer to the well-coagulating samples. Figure 2A shows a plot of the most important components reflecting 91.2% of the total variation [principal component (**PC**)1: 86% and PC2: 5.2%].

Subsequently, only the well-coagulating and noncoagulating milk samples were analyzed by PCA. The results (Figure 2B) showed 2 distinct clusters. Well-coagulating and noncoagulating milk spectra were separated by PC2 (y-axis), with all well-coagulating samples positioned above a threshold, and all noncoagulating samples below this threshold. Principal component 1 (x-axis) and PC3 did not differentiate between these 2 groups significantly. Thus, we concluded that, in addition to other sources of variation, a specific signature exists for well-coagulating milk compared with noncoagulating milk. The same outcome was observed for well-coagulating and noncoagulating milk samples from the SCC-corrected database (Figure 2C).

Heat-Map Analysis. To display the distribution of signal intensities relatively to the mean at respective m/z values, a heat-map analysis was produced (Figure 3). A heat-map is a structured display of data where different colors represent different expression levels of the metabolites. The m/z values were first ranked based on the calculated statistical differences between the signal intensities in the noncoagulating and well-coagulating groups, and only the most significant differences are shown. Figure 3 shows that alterations in both directions were present: some compounds were more abundant in well-coagulating milk and others in noncoagulating milk. Signals with a difference of 1 or 2 amu, and with a very similar pattern among samples, are likely to be isotopes of the same substance. Even considering this, the number of markers found was not limited to a small number of metabolites, indicating that more than a single compound is involved in the reduced coagulation capacity of some milks. In the intermediate group, samples with poor coagulation ability were more similar to noncoagulating milk, whereas most of the others were more similar to the well-coagulating milk samples. To further illustrate this, the heat-map analyses were carried out separately on the intermediate group (Figure 3B). A difference was observed even among the samples with poor and good coagulation abilities. Heat-map analysis was also carried out on the SCC-corrected database, comparing noncoagulating and well-coagulating milk samples. Figure 3C shows the 25 m/z values most significantly different within this data set. Although the noncoagulating and well-coagulating milks were significantly different ($P \leq 0.05$), the intermediate group milks were not significant. Compounds with m/z 534, 601, and 162 were represented on all heat-maps; signals were more intense in samples with better coagulation ability.

Correlation Analyses

Coagulation analyses were carried out on all 143 milk samples, and coagulation time was found to be positively correlated with milk pH (R = 0.48). Curd firmness had a highly negative correlation with pH (R = −0.80) and coagulation time and curd firmness were negatively correlated (R = −0.58). Okigbo et al. (1985) also noted that coagulation time was positively correlated with milk pH (R = 0.58); curd firmness had a negative correlation (R = −0.53) with pH; and coagulation time and curd firmness were highly negatively correlated (R = −0.86). Ikonen et al. (2004) and Kübarsepp et

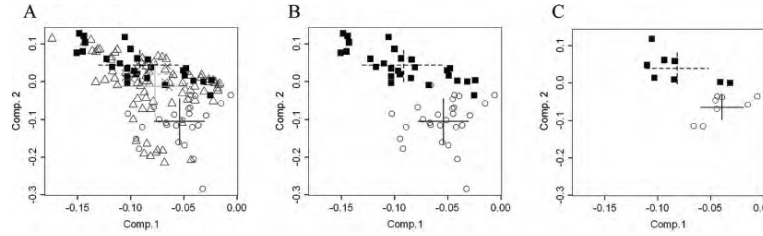


Figure 2. Principal component (comp.) analyses (PCA) of all 143 milk samples: (A) well- (■), poor (Δ), good (×), and noncoagulating (○) samples; (B) PCA of well- (■) and noncoagulating (○) milk samples; (C) PCA of SCC-corrected data set of well- (■) and noncoagulating (○) milk samples was used. Crosses represent the mean ± SEM.

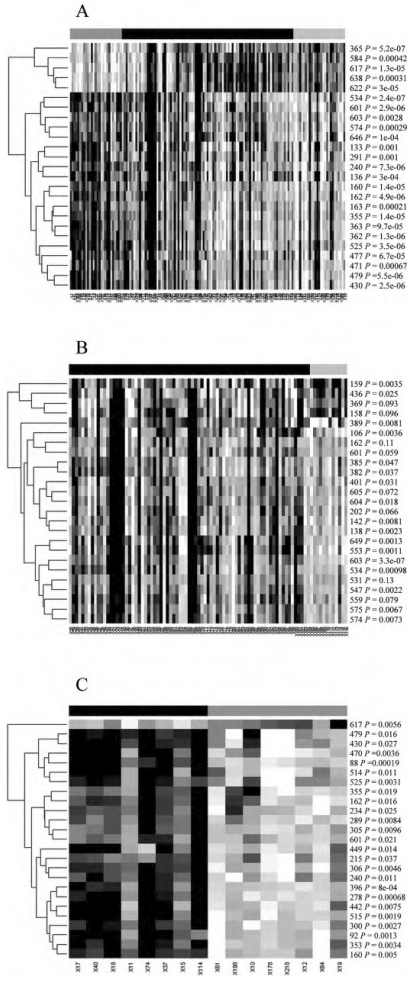


Figure 3. (A) Heat map of noncoagulating (dark gray bar on top left), intermediate (black bar on top center), and well-coagulating (light gray bar on top right) milk samples. Statistical comparison with Student *t*-test was calculated between well-coagulating and noncoagulating groups. The 25 most significantly different m/z values with respective *P*-values can be seen in the right-hand side. Rows are arranged into clusters (dendrogram on left-hand side) based on pattern similarities. The lighter the shading of a data point, the more intense the signal is compared with the mean value for the respective m/z among all analyses. Gray points are close to the mean and dark tones indicate signal intensities below the mean; (B) heat map of milks with poor (black bar) and good (gray bar) coagulation ability; (C) heat map of noncoagulating (black bar) and well-coagulating (gray bar) milks.

al. (2005b) found identical (in the same direction) but weaker phenotypic correlations in their large-scale studies covering a few thousand samples than were found in the current study. The correlation between coagulation time and milk pH was moderate (R = 0.29 and R = 0.39, respectively), as was the correlation between SCS and milk pH (R = 0.26 and R = 0.19, respectively). Milk pH had weak negative correlation with curd firmness (R = −0.18 and R = −0.15, respectively).

No significant correlations were found between curd firmness, MS signals, and herd or breed data, perhaps because of the relatively large number of herds compared with the total number of samples. With simultaneous comparison of 39 herds, the putative herd-specific markers might cancel each other out as noise in biological variance. The accompanied increase in noise may also make breed-specific markers harder to detect.

It has been suggested that the poor coagulation ability of milk can be predicted based on SCC and milk pH (Grandison and Ford, 1986; Ikonen et al., 2004); no known factors independently cause the noncoagulation of milk. In addition to correlating classical milk characteristics with each other and with coagulation ability, we wanted to know whether SCS, pH, coagulation ability, or E_{30} were correlated with LC-MS/MS signals. The strongest negative correlation (R = −0.30) was found between SCS and $m/z = 535$ (Table 2), and the highest positive correlation (R = 0.61) between SCS and $m/z = 1{,}110$. From the lower mass range under m/z 499, no significant correlation with SCS was found. In general, SCS was weakly correlated (R = 0.28) with the discovered markers. The most positive correlation with SCS was to signals with an m/z of over 1,000. The mass range, and fact that many signals showed similar correlations, suggests that a high SCC causes higher

Table 2. Correlation coefficients of SCS, pH, and curd firmness (E_{30}) with markers identified by m/z

m/z	SCS	pH	E_{30}
160	−0.01	−0.15	0.20
162	−0.05	−0.29	0.34
240	0.09	−0.05	0.18
362	−0.09	−0.04	0.12
365	0.17	0.34	−0.18
430	−0.00	−0.17	0.25
479	−0.06	−0.23	0.31
525	−0.02	−0.02	0.06
534	−0.29	−0.44	0.44
535	−0.30	−0.47	0.48
584	0.50	0.41	−0.36
601	0.16	−0.08	0.17
617	0.48	0.44	−0.39
622	0.53	0.31	−0.28
638	0.47	0.36	−0.27
851	0.54	0.64	−0.52
1,046	0.49	0.63	−0.53
1,110	0.61	0.45	−0.40

background noise from peptides and oligosaccharides. The strongest negative correlation (R = −0.47) was between pH and $m/z = 535$, and the most positive correlation between pH and $m/z = 851$ (R = 0.64). No significant correlations (R = −0.08) were found between milk pH and the identified markers in the mass range under m/z 499. A moderate positive correlation (R = 0.29) was noted in the mass range of m/z 500 to 999, and a stronger positive correlation (R = 0.40) in the mass range of m/z 1,000 to 1,500.

Analysis of Markers

Signals with significant differences between well-coagulating and noncoagulating milks were selected for further analysis (Figure 3A, B, and C). First, to specify the molecular weights, ion charge (z) values were determined (Table 3). Surprisingly, a tendency was observed for the signals that were stronger in well-coagulating milks to be doubly charged. Signals from the noncoagulating milks had a value of $z = 1$. With the values of charges known, molecular weights were calculated and most were observed to be above 500 amu. Nonpolymeric compounds rarely exceed a molecular weight of 600 amu (Kind and Fiehn, 2007). Masses around m/z 1,000 could indicate lipids, peptides, or oligosaccharides. The hydrophobicity of the selected markers was assessed by C_{18} and HILIC columns. None of the markers had significant interactions with the C_{18} analytical column, implying a lack of hydrophobicity. From the HILIC column, the compounds eluted after 27 min (Table 3).

Fragmentation analysis was carried out and results were compared with the spectra in the human metabolome database (htttp://www.hmdb.ca). A signal of m/z = 162 was found to match carnitine, but no defined match was found for the others. Yet it was noted that the spectra shared similarities. The first common characteristic was a repetitive loss of 18 amu; second, fragments with m/z values of 99, 180, and 261 were shared by many compounds; and third, compounds with higher mass yielded fragments matching molecular weights of smaller compounds. Taken together, the fragmentation spectra imply the presence of oligosaccharides. This was further tested by determining fragmentation and HILIC retention times of glucose, sucrose, lactose, and *N*-acetyllactosamine (Table 3). Beside protonated molecular ions, sodium and potassium adducts and $[M\text{-}H_2O]^+$ ions were observed. Signal $m/z = 365$, found in commercial *N*-acetyllactosamine (Sigma-Aldrich) and believed to represent $[M\text{-}H_2O]^+$ ion, yielded fragments

Table 3. Characteristics of mass spectrometry signals that have most significant differences between well-coagulating and noncoagulating milks

Item	z[1]	RT[2] in HILIC[3] column (min)	Main fragments in MS/MS analysis (m/z)[4]	Comment
Signals higher in noncoagulating milks (m/z)				
365	1	27.6	305, 203, 347, 245, 185, 83	NAcLac – H_2O[5]
617	1	26.4	261, 417, 542, **365**, 451, 86	
638	1	26.5	296, **365**, 261, 416, 226, 104	
622	1	26.5	280, 221, 104, 129, **365**, 86	
584	ND[6]	25.7	261, 187, 242, 129, **365**, 70	
Signals higher in well-coagulating milks				
479	1	8.6	359, 261, 137, 99, 125, 197	
430	2	5.6	260, 99, 180, 150, 231, 310	
525	2	26.3	354, 345, 245, 174, 305, 97	
355	2	7.9	247, 175, 97, 169, 85, 157	
362	2	28.1	99, 182, 261, 201, 97, 111	
534	2	27.9	362, 182, 353, 261, 191, 201	
601	2	25.7	260, 310, 321,370, 180, 231	
160	1	9.7	101, 77, 60, 119, 58, 83	
162	1	11.2	103, 102, 121, 85, 139, 60	Carnitine
240	1	10.4	99, 111, 125, 153, 201, 97	
Control compounds				
384	1	27.0	138, 204, 186, 168, 126, 144	NAcLac
343	1	27.5	85, 97, 127, 91, 109, 145	Lactose
181	1	23.9	99, 139, 161, 120, 85, 163	Glucose
343	1	27.1	163, 85, 127, 145, 97, 260	Sucrose
365	1	27.0	347, 305, 203, 245, 185	NAcLac – H_2O

[1]Ion charge.
[2]Retention time (min).
[3]Hydrophilic interaction liquid chromatography.
[4]The repeating fragment of $m/z = 365$ is in bold.
[5]*N*-acetyllactosamine as the $[M\text{-}H_2O]^+$ ion.
[6]For one compound, no clear isotopic distribution could be obtained and determination of z was therefore not possible (ND).

that matched well with an unknown signal (m/z = 365). Because of the structural similarities of various saccharides, this identification should be regarded with caution. Interestingly, in all of the 4 signals that were elevated in noncoagulating milks, a fragment with m/z = 365 was observed. No marker from the well-coagulating milk yielded such a fragment. Further analysis to determine the exact oligosaccharides affecting coagulation ability are necessary.

CONCLUSIONS

This study found correlations between milk technological properties (in this case, coagulation) and its metabolome. This study confirmed that such correlations exist and could open the way to improve these technological properties through animal nutrition or selection, which could influence the level of the identified metabolites. Our results show that it is possible, using MS analyses, to detect signals that correlate with either the coagulation capacity or other parameters (e.g., pH and SCC) believed to influence the coagulation process. It is not yet possible to describe in detail the mechanism whereby low molecular weight compounds enhance or inhibit coagulation, but the results presented represent a first step in this direction.

ACKNOWLEDGMENTS

This research was carried out by the Bio-Competence Centre of Healthy Dairy Products and was co-financed by the European Community's Regional Development Fund within the framework of the Competence Centre Program of the Enterprise Estonia, project no. EU30002, targeted finance project SF0170165s08, and Estonian Science Foundation grants 7494 and 7856.

REFERENCES

Auldist, M., C. Mullins, B. O'Brien, B. T. O'Kennedy, and T. Guinee. 2002. Effect of cow breed on milk coagulation properties. Milchwissenschaft 57:140–143.

Cassandro, M., A. Comin, M. Ojala, R. Dal Zotto, M. De Marchi, L. Gallo, P. Carnier, and G. Bittante. 2008. Genetic parameters of milk coagulation properties and their relationships with milk yield and quality traits in Italian Holstein cows. J. Dairy Sci. 91:371–376.

De Marchi, M., R. Dal Zotto, M. Cassandro, and G. Bittante. 2007. Milk coagulation ability of five dairy cattle breeds. J. Dairy Sci. 90:3986–3992.

Grandison, A. S., and G. D. Ford. 1986. Effects of variations in somatic-cell count on the rennet coagulation properties of milk and on the yield, composition and quality of cheddar cheese. J. Dairy Res. 534:645–655.

Guinee, T. P., E. O. Mulholland, B. O'Brien, and J. J. Murphy. 2001. Effect of diet quality on the suitability of mid-lactation bovine milk for cheddar cheese manufacture. Aust. J. Dairy Technol. 56:3–8.

Harmon, R. J. 1994. Physiology of mastitis and factors affecting somatic cell counts. J. Dairy Sci. 77:2103–2112.

Hooydonk, A. C. M., I. J. Boerrigter, and H. G. Hagedoorn. 1986. pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 2. Effect of pH on renneting of milk. Neth. Milk Dairy J. 40:297–313.

Ikonen, T., S. Morri, A. M. Tyrisevä, O. Ruottinen, and M. Ojala. 2004. Genetic and phenotypic correlations between milk coagulation properties, milk production traits, somatic cell count, casein content, and pH of milk. J. Dairy Sci. 87:458–467.

Ingvartsen, K. L. 2006. Feeding- and management-related diseases in the transition cow—Physiological adaptations around calving and strategies to reduce feeding-related diseases. Anim. Feed Sci. Technol. 126:175–213.

Jõudu, I. 2008. Effect of milk protein composition and genetic polymorphism on milk rennet coagulation properties. PhD Thesis. Estonian University of Life Sciences, Tartu. http://dspace.emu.ee/jspui/bitstream/10492/113/1/Thesis_Joudu_2008.pdf.

Kelly, A. L. 2003. Milk Quality and Udder Health. Test Methods and Standards. Pages 1995–2002 in Encyclopedia of Dairy Sciences. Vol. 3. H. Roginski, J. W. Fuquay, and P. F. Fox, ed. Academic Press, London, UK.

Kind, T., and O. Fiehn. 2007. Seven Golden Rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. BMC Bioinformatics 8:105–125.

Kübarsepp, I., M. Henno, O. Kärt, and T. Tupasela. 2005a. A comparison of the methods for determination of the rennet coagulation properties of milk. Acta Agric. Scand. Anim. Sci. 55:145–148.

Kübarsepp, I., M. Henno, H. Viinalass, and D. Sabre. 2005b. Effect of κ-casein and β-lactoglobulin genotypes on the milk rennet coagulation properties. Agron. Res. 3:55–64.

Malossini, F., S. Bovolenta, C. Piras, M. DallaRosa, and W. Ventura. 1996. Effect of diet and breed on milk composition and rennet coagulation properties. Ann. Zootech. 45:29–40.

McCarthy, O. J., and H. Singh. 2009. Physicochemical properties of milk. Pages 691–758 in Advanced Dairy Chemistry: Volume 3: Lactose, Water, Salts, and Minor Constituents. P. L. H. McSweeney and P. F. Fox, ed. Springer, London, UK.

O'Brien, B., W. J. Meaney, D. McDonagh, and A. Kelly. 2001. Influence of somatic cell count and storage interval on composition and processing characteristics of milk from cows in late lactation. Aust. J. Dairy Technol. 56:213–218.

Okigbo, L. M., G. H. Richardson, R. J. Brown, and C. A. Ernstrom. 1985. Variation in coagulation properties of milk from individual cows. J. Dairy Sci. 68:822–828.

Ostersen, S., J. Foldager, and J. E. Hermansen. 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. J. Dairy Res. 64:207–219.

R Development Core Team. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Rohner-Thielen, E. 2008. From grass to glass: A look at the dairy chain. Eurostatistics 76:1–8.

Tyrisevä, A. M., K. Elo, A. Kuusipuro, V. Vilva, I. Janonen, H. Karjalainen, T. Ikonen, and M. Ojala. 2008. Chromosomal regions underlying noncoagulation of milk in Finnish Ayrshire cows. Genetics 180:1211–1220.

Tyrisevä, A. M., T. Ikonen, and M. Ojala. 2003. Repeatability estimates for milk coagulation traits and non-coagulation of milk in Finnish Ayrshire cows. J. Dairy Res. 70:91–98.

Tyrisevä, A. M., T. Vahlsten, O. Ruottinen, and M. Ojala. 2004. Noncoagulation of milk in Finnish Ayrshire and Holstein-Friesian cows and effect of herds on milk coagulation ability. J. Dairy Sci. 87:3958–3966.

Vallas, M., H. Bovenhuis, T. Kaart, K. Parna, H. Kiiman, and E. Parna. 2010. Genetic parameters for milk coagulation properties in Estonian Holstein cows. J. Dairy Sci. 93:3789–3796.

Wedholm, A., L. B. Larsen, H. Lindmark-Mansson, A. H. Karlsson, and A. Andren. 2006. Effect of protein composition on the cheesemaking properties of milk from individual dairy cows. J. Dairy Sci. 89:3296–3305.

II

Ilves, A., **Harzia, H.,** Ling, K., Ots, M., Soomets, U., Kilk, K. 2012
ALTERATIONS IN MILK AND BLOOD METABOLOMES
DURING THE FIRST MONTHS OF LACTATION IN DAIRY COWS
Journal of Dairy Science, 95(10), 5788 - 5797

J. Dairy Sci. 95:5788–5797
http://dx.doi.org/10.3168/jds.2012-5617

Alterations in milk and blood metabolomes during the first months of lactation in dairy cows

A. Ilves,*†[1] H. Harzia,*† K. Ling,* M. Ots,* U. Soomets,† and K. Kilk†
*Department of Nutrition and Animal Products Quality, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, 46 Kreutzwaldi St., 51006, Tartu, Estonia
†Department of Biochemistry, Medical Faculty, University of Tartu, the Centre of Excellence for Translational Medicine, 19 Ravila St., 50411, Tartu, Estonia

ABSTRACT

The molecular composition of milk is influenced by various genetic and environmental factors. Time is one important factor, and the fact that certain milk components change over the course of lactation is widely accepted. Untargeted global metabolomics is an approach to study hundreds of low molecular weight compounds simultaneously. In this study, mass spectrometry-based global metabolomics was used to follow the course of changes in milk (n = 133) and blood plasma (n = 133) during the early stage of lactation. Little correlation was found between the molecular composition of blood plasma and milk. Blood showed a higher dependence on animal individuality than did milk, in which common evolutions in time resolved. Citrate and lactose had the greatest effect on these changes; however, the most significant changes in milk during the first months of lactation were associated with phosphorylated saccharide levels, whereas the most significant changes in blood plasma were associated with levels of polyunsaturated fatty acids containing phosphatidylcholine. In conclusion, a new systemic approach was used to search for minor metabolites whose concentrations were significantly altered in milk and blood during the first months of lactation.
Key words: dairy cow, early lactation, milk and blood metabolome, tandem mass spectroscopy

INTRODUCTION

Milk is a complex colloidal solution of different macroconstituents synthesized in the mammary gland, and microconstituents originating mostly from blood. It is well known that the concentrations of the main constituents in dairy cow milk differ between breeds (DePeters et al., 1995), stages of lactation (Waite et al., 1956), and composition of the ration (Elgersma et al., 2004). It is acknowledged that over the course of lactation, milk composition changes per se, although the most significant changes occur in early and late lactation. During midlactation, the composition of milk remains relatively constant (Fox, 2009).

A greater fat-to-protein ratio (Heuer et al., 2000) and elevation of concentrations of ketone bodies, such as acetone, acetoacetate, and BHBA, in milk are related to the mobilization of body reserves during postpartum energy deficiency (Nielsen et al., 2003). Milk citrate concentration is also higher in early lactation and is related to de novo synthesis of FA (Garnsworthy et al., 2006). Feeding also has an effect on the fat-to-protein ratio (Grant et al., 1990; He and Armentano, 2011) as well as on the urea nitrogen content in milk (Nousiainen et al., 2004).

In blood, energy metabolite profiles, such as NEFA, BHBA, triglyceride, glucose, and insulin, already begin to change by the end of gestation and continue to change during the first weeks of lactation (Holtenius et al., 2003; Bossaert et al., 2008). All these compounds are used to evaluate the metabolic status of a cow.

Metabolomics is the detection of low molecular weight metabolites and their intermediates from biofluids or tissues. It is used widely in many fields, such as pharmacology, toxicology, and diagnostics, and its use and technological development have increased rapidly (Rochfort, 2005; Zhang et al., 2012). So far, however, few data have been available on the milk and blood metabolomes of dairy cows. Boudonck et al. (2009) identified 223 metabolites from 10 different commercially available milk samples, which showed high biochemical variability attributable to the different manufacturing procedures. Klein et al. (2010), who studied the differences in milk metabolites in samples from early, mid, and late lactation, quantified 44 different milk metabolites. Their results confirmed that milk acetone and BHBA levels are positively correlated with the metabolic status of individual cows in early lactation.

Received April 13, 2012.
Accepted June 12, 2012.
[1]Corresponding author: aire.ilves@emu.ee

Table 1. Ingredients and chemical composition of the TMR

Item	Amount (g/kg of DM)
Ingredient	
Grass silage 1	280
Grass silage 2	104
Barley (crushed)	292
Rapeseed cake	180
Corn meal	124
Mineral mix[1]	10
Salt	4
Limestone[2]	6
Chemical composition	
ME (MJ)	11.4
CP	160.1
MP	103.8

[1]Ingredients: calcium, 115 g/kg; phosphorous, 45 g/kg; magnesium, 140 g/kg; sodium, 90 g/kg.
[2]Calcium, 370 g/kg.

In addition, higher concentrations of α-aminobutyric acid, phosphocholine, and glycine during the first third of lactation were identified. In a more recent study with nuclear magnetic resonance metabolomic analysis, Klein et al. (2012) found that the ratio of glycerophosphocholine to phosphocholine during the first 4 wk of lactation and the high concentration of glycerophosphocholine at midlactation may serve as markers to identify metabolically stable and healthy cows. In addition, no significant correlations between milk and blood plasma metabolites levels were found within the first 5 wk of lactation.

Nuclear magnetic resonance is 1 of the 2 major platforms used in metabolomics. Because of different signal detection principles, nuclear magnetic resonance and mass spectrometry results are not directly comparable. Nuclear magnetic resonance has certain advantages over mass spectrometry, such as noninvasiveness, but at the same time, it suffers from lower sensitivity (Roux et al., 2011). The aim of this study was to investigate low molecular weight metabolites and compare the molecular composition of milk and blood plasma during the first months of lactation with untargeted global metabolomics by using tandem mass spectrometry.

MATERIALS AND METHODS

Animal Management and Feeding

Animal use and care was in accordance with the Estonian Animal Protection Act. Cows in a loose housing system at the Estonian University of Life Sciences experimental farm (Eerika Farm LLC, Märja, Estonia) were fed a TMR (Table 1) ad libitum twice a day. The basal diet (60% concentrates, 40% silage) consisted of grass silage, barley meal (crushed), rapeseed cake, corn meal, limestone, salt, and a mineral mix for lactating cows. Feed values of the TMR were determined by methods described by the AOAC Int. (2005) and were calculated monthly on the basis of the chemical composition of the ingredients. Cows were milked twice a day, and milk yield was recorded daily. At the beginning of lactation, the mean BW of the 5 clinically healthy multiparous (second- to fifth-parity) Estonian Holstein cows (Table 2) was 642.5 ± 27.3 kg; their mean ECM yield over the study period (5.6 ± 0.5 to 96.2 ± 6.6 DIM) was 43.7 kg (range of 40.1 to 48.2).

Sample Collection

Milk samples (n = 133) were collected twice a week (October 2009 to April 2010) with in-line milk meters, and samples were stored at −20°C until analyzed for metabolites. Blood samples (n = 133) from the coccygeal vein (Venoject vacuum tubes, Terumo Europe

Table 2. Data on lactation, DMI, ECM, energy balance, and BCS for the cows

	Cow				
Item	A	B	C	D	E
Lactation	5	3	3	3	2
DMI[1] (kg/d)	23.5	24.1	25.8	24.2	26.9
ECM[1,2] (kg/d)	45.2	45.8	48.2	40.1	42.2
Nadir of negative EB[3] (DIM)	11	21	24	10	10
Nadir of negative EB[3] (MJ/d)	−207	−163	−147	−161	−61
Start of positive EB[3] (DIM)	48	72	56	26	19
BCS[4] before calving	3.50	3.50	3.75	3.75	3.50
BCS[4] at end of study period	2.50	2.50	2.75	2.75	2.75

[1]Mean for the study period.
[2]ECM as calculated by Sjaunja et al. (1990).
[3]EB = calculated energy balance (EB = $E_{consumed} - E_{maintenance} - E_{lactation}$).
[4]BCS as measured by Edmonson et al. (1989).

N.V., Leuven, Belgium) were collected after the evening milking on the milk sampling days. Plasma was separated by centrifugation (4,500 × *g*, 15 min, 22°C) immediately after sampling and kept at −20°C until analyzed.

Mass Spectrometry Analyses

The milk samples for mass spectrometry analyses were prepared and analyzed, as described by Harzia et al. (2012), with a hybrid triple quadrupole/linear ion trap tandem mass spectrometry system (3,200 Q TRAP; AB Sciex Instruments, Foster City, CA). Positive and negative ions (50 to 1,700 Da) were measured with a TurboIonSpray ion source (AB Sciex Instruments, Foster City, CA) using an enhanced mass scan. For compound identification, C18 (Luna 3-μm C18 100A, 100 × 2.00 mm; Phenomenex, Torrance, CA) and HILIC (Luna 5-μm HILIC 200A, 150 × 3.00 mm; Phenomenex) columns were used. The system was controlled by Analyst software version 1.4.2 (Applied Biosystems Inc., Foster City, CA).

Statistical Analyses

To find patterns in the data with a high dimension, principal components analysis (**PCA**) was used with R-Statistics software version 2.8.1. (BioConductor algorithms; R Development Core Team, 2008). Analysis of variance and the Tukey test were performed to test for significant differences between mean mass-to-charge ratio values. A volcano plot, a scatter plot analysis, was plotted to visualize the change in mean signal intensity versus the statistical significance of the change.

RESULTS AND DISCUSSION

Principal components analysis is a method to compress data in large matrixes and classify samples based on variations between them. Therefore, PCA was used for preliminary comparison of the mass spectra of milk and blood plasma samples. For each cow, an "evolution" (i.e., directed movement in the plot of principal components) was observed in the milk metabolic profile of negatively charged ions during lactation (Figure 1). Depending on the animal, samples up to 21 to 30

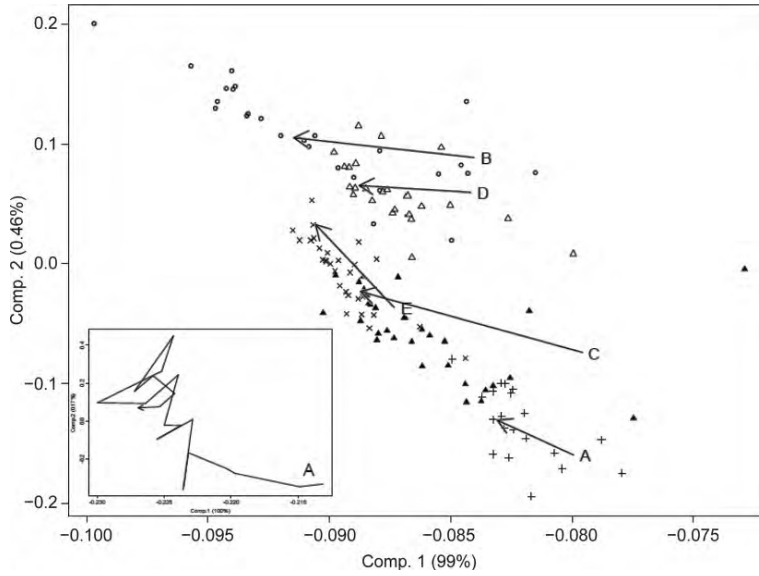


Figure 1. Principal components analysis of milk metabolomes. Each dot represents an entire spectra of negatively charged ions within mass-to-charge ratio (m/z) 50 to 1,500. The arrow begins from the mean coordinates of the first 5 samples (5 to 20 DIM) and ends at the mean coordinates of the last 5 samples (58 to 74 DIM). Letters A to E indicate individual animals. The inset shows the path from each time point to the next time point for animal A. Comp. = component.

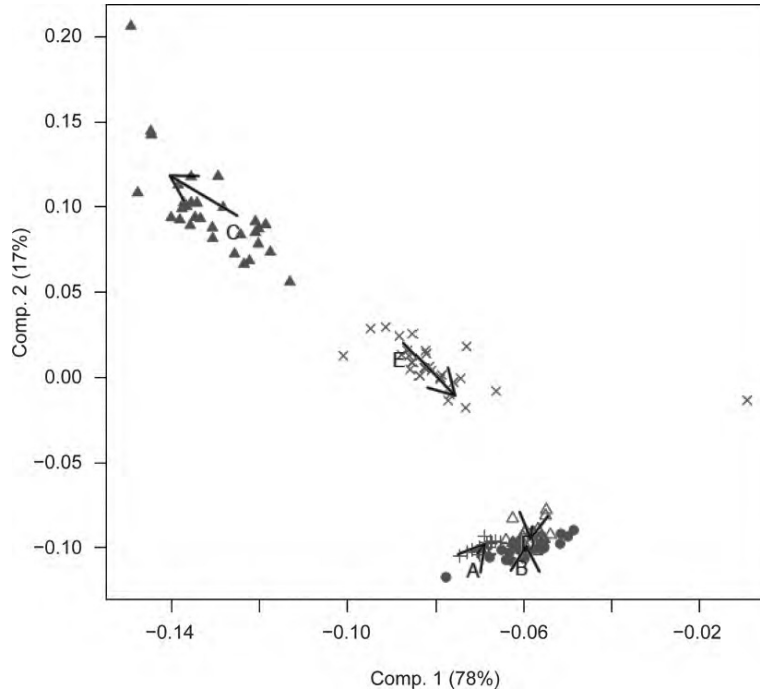


Figure 2. Principal components analysis of blood plasma metabolomes. Each dot represents an entire spectra of negatively charged ions within mass-to-charge ratio (m/z) 50 to 1,500. The arrow begins from the mean coordinates of the first 5 samples (5 to 20 DIM) and ends at the mean coordinates of the last 5 samples (58 to 74 DIM). Letters A to E indicate individual animals. Comp. = component.

DIM were those classified by PCA as determining the evolution and were separated from the samples from subsequent DIM. Global metabolite spectra from samples beyond the first lactation month showed lower variability, implying less significant changes after that time point. The differences between the individual animals make their direct comparison and averaging of the results difficult. Figure 1 shows that, despite the different starting and end points, the direction of evolution from the first to the second month of lactation is common. This implies that the milk metabolites behind the shift are the same in all cases. Loading plots suggested signals with m/z 117, 119, 121, 161, 163 (all identified as saccharides or fragments thereof), 191 (citrate), 341 (lactose), and 377 (unknown) as the main contributors to the 2 highest principal components. On the basis of fragmentation spectra, the saccharides contained no phosphate or sulfate groups. In positive ionization mode spectra, the highest variance showed no common evolution, yet lower spectra (components 4 and 5, associated with saccharides) did. Therefore, the concentration of preferably positively charged low molecular weight metabolites depended on other factors more than on the lactation stage. Exclusion of the first month time points from the analysis removed the universal direction of evolution, implying that from the second month of lactation, changes in milk composition were minor or individual traits, or both.

The PCA of blood plasma metabolic profiles in the negative mode showed no universal evolution; the patterns of individual animals (A to E) were different (Figure 2), and samples from 2 animals (C and E) were classified separately from the other 3. Principal components analysis loading plots, ANOVA, and the Tukey test were used to find mass-to-charge ratio values characteristic for each animal (Table 3). Animals A, B, and D had 28, 15, and 26 signals, respectively, with a P-value of <0.0001, which is the estimated metabolome-

Table 3. Animal (A–E)-specific markers in blood plasma[1]

	A		B		C		D		E	
Item	m/z	P-value	m/z	P-value	m/z	P-value	m/z	P-value	m/z	P-value
Negative ions										
	312	2.6E-11	187	1.5E-12	60	2.0E-14	265	2.2E-14	168	2.0E-14
	161	7.9E-10	179	6.9E-09	62	2.0E-14	266	3.1E-14	60	2.2E-14
	163	3.7E-09	234	1.4E-08	128	2.0E-14	115	8.7E-13	179	2.3E-14
	165	5.9E-09	178	4.8E-08	194	2.0E-14	535	7.0E-08	95	2.5E-14
	159	9.5E-09	134	6.4E-08	196	2.0E-14	307	8.8E-07	93	2.5E-14
	384	1.0E-08	212	1.1E-07	198	2.0E-14	103	1.3E-06	677	2.5E-14
	135	3.1E-08	236	3.8E-07	199	2.0E-14	590	1.5E-06	693	2.5E-14
	137	1.6E-07	215	1.0E-06	200	2.0E-14	478	3.2E-06	712	2.5E-14
	315	3.3E-07	733	1.3E-05	202	2.0E-14	594	4.5E-06	675	2.6E-14
	313	7.1E-07	216	4.7E-05	206	2.0E-14	299	5.4E-06	681	2.6E-14
Positive ions										
	151	5.6E-06	503	1.4E-07	100	2.0E-14	171	2.4E-08	502	1.4E-10
	478	7.7E-05	157	3.4E-06	119	2.0E-14	157	7.9E-07	478	3.5E-10
	165	3.9E-04	192	9.5E-05	121	2.0E-14	89	2.2E-04	120	7.2E-09
	224	7.0E-04	1,307	1.6E-03	122	2.0E-14	1,232	1.5E-03	1,379	1.1E-08
	81	3.8E-03	259	4.6E-03	128	2.0E-14	120	1.5E-02	309	1.5E-08
	149	3.8E-03	72	6.5E-03	156	2.0E-14	72	2.0E-02	504	2.1E-08
	185	4.0E-03	191	7.0E-03	205	2.0E-14	506	2.2E-02	506	2.8E-08
	199	5.3E-03	508	9.7E-03	207	2.0E-14	172	3.7E-02	95	3.6E-08
	257	7.1E-03	224	1.1E-02	225	2.0E-14	504	4.5E-02	1,380	4.2E-08
	120	8.1E-03	260	1.6E-02	226	2.0E-14	478	5.4E-02	1,399	5.8E-08

[1]Mass-to-charge ratios (m/z) for the 10 most significant signals for negatively and positively charged ions are listed. Molecular species behind these signals were not identified (for comments see text).

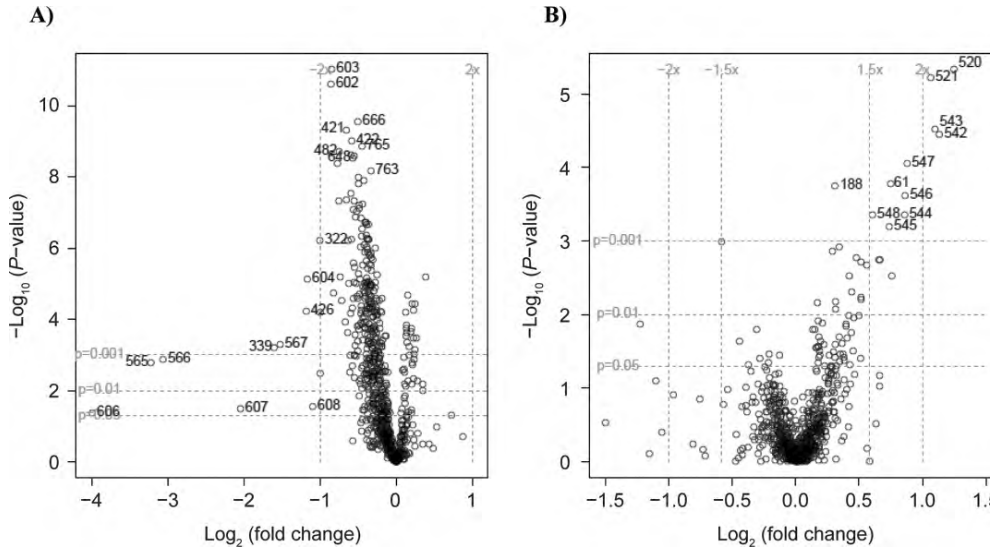


Figure 3. Volcano plots displaying the fold change in mass-to-charge ratio intensity (x-axis) versus the significance of the change (y-axis). Signals with the highest and most significant increase in the first 2 mo of lactation are in the upper right corner. Signals with the highest and most significant decrease in time are in the upper left corner of each figure. A) Negatively charged ions from milk; B) positively charged ions from plasma. Among the positive ions from milk and the negative ions from plasma, no significant changes were observed, so these graphs are omitted.

wide significance level for the current setup (Chadeau-Hyam et al., 2010). Animal C had 806 and animal E had 752 signals. However, the enormous number of altered metabolites may be a matrix-related effect, such as ion suppression. High-abundance compounds in a complex mixture may interfere with ionization of other compounds, suppressing detected signal intensities. A major contributor in differentiating animals A, B, and D from C and E was a group of structurally related hydrophilic compounds with m/z 161, 163, 196, 198, 200, and 202. On the basis of intensity, the compounds represented by these signals had high concentrations in plasma and might lead to a background shift in mass spectra and false positive changes in the intensities of unrelated signals. In a comparison of animals A, B, and D, in which the matrix effects were not as striking, animal A had an increase in a group of related signals with m/z 137, 312, 313, 315, and 384. These metabolites were found to be very hydrophobic and could be monoglycerides or FA esters with glycine. Signals m/z 265, 266, and 115 in animal D were found to have no correlations with other signals; that is, the activities of related metabolic pathways were not significantly affected by the concentration of these compounds, or they were xenobiotics with no related pathways.

The next question was how large and significant the changes were in time. Therefore, volcano plots were created visualizing the change in mean signal intensity versus the statistical significance of the change. Pooling data from all animals showed that the most significantly changed mass-to-charge ratio values in milk were m/z 601 to 603, 421 to 422, 666, 482, and

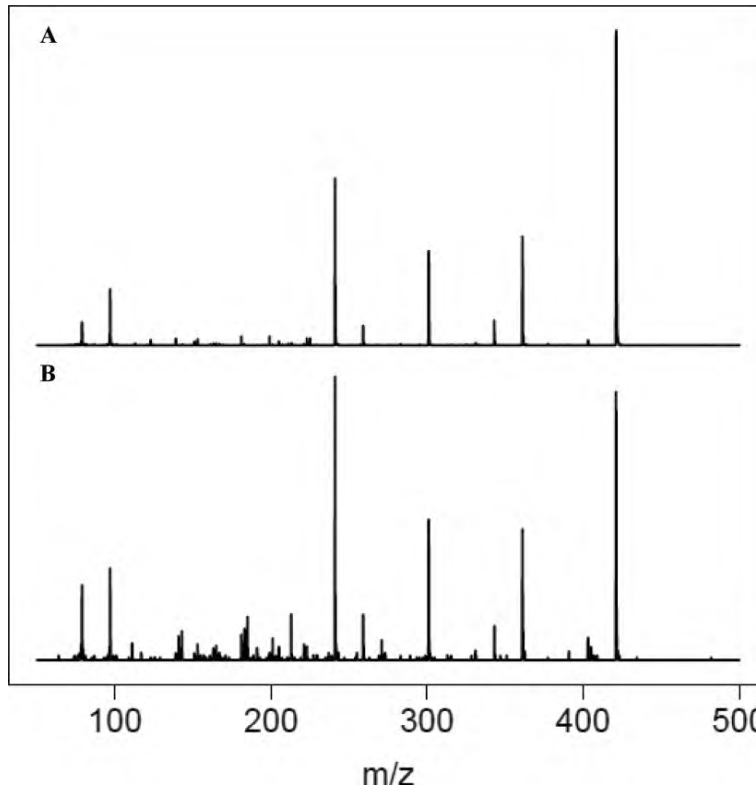


Figure 4. Fragmentation spectra of mass-to-charge ratio (m/z) 421 from a solution of commercial lactose phosphate (A) and from milk samples (B).

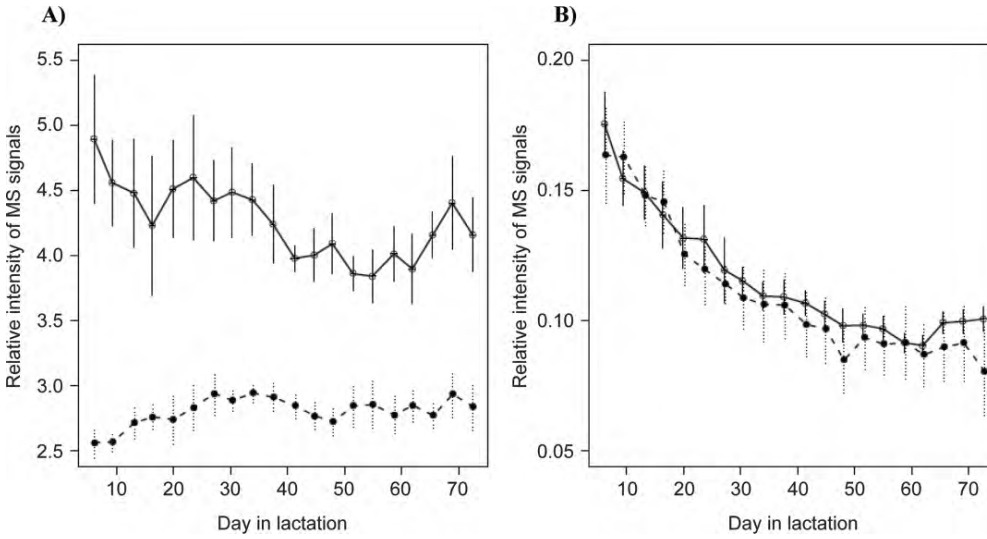


Figure 5. Change in relative signal intensity in milk over the first months of lactation. A) Citrate (solid line), lactose (dashed line); B) lactose phosphate (solid line), mass-to-charge ratio (m/z) 601, unknown phosphosaccharide (dashed line). MS = mass spectrometry.

763 to 765 (negative ions, $P < 10^{-8}$; Figure 3A). All these signals decreased by the start of the third lactation month to 75 to 60% of their initial values. Signals m/z 565 to 566 and 606 to 607 were found to have a more pronounced decrease in time, but with lower significance. Fragmentation analysis revealed a high similarity of m/z 421 with lactose phosphate (Figure 4). Signals m/z 601 to 603 and 763 to 765 gave only 2 detectable fragments. Both matched signals in the m/z 421 spectra. The difference of 162 amu between m/z 763 and 601 suggests an additional hexose in 763 compared with 601. The signal 601 itself is 180 amu more than 421, suggesting a noncovalent complex with additional hexose or a covalent hexose + water. Although the structures of saccharides are difficult to determine by mass spectrometry, these data imply that the substances most significantly decreasing during the first one-third of lactation are phosphorylated saccharides. In an early study, McGeown and Malpress (1952) detected phosphate esters of galactose and lactose in skim milk, with the lactose phosphate concentration being less than 0.1 mg/L. In a later study, Petzold et al. (2004) identified 2 disaccharides with m/z 421 in bovine colostrum: a phosphorylated dihexose, Hex2P, with m/z 421.0656, and a sulfated dihexose, Hex2S, with m/z 421.0756. As in the current study, Petzold et al. (2004) found phosphorylated disaccharides in higher abundance. However, taking into account the results of Petzold et al. (2004), it is not possible to exclude the possibility of the involvement of sulfated disaccharides in signal m/z 421 in the current study.

According to PCA, citrate and unphosphorylated saccharides (lactose) contributed the most to the milk metabolome evolution during early lactation (Figure 5). Considering the role of citrate in providing reducing equivalents for de novo synthesis of FA in the mammary gland (Garnsworthy et al., 2006), the decrease in its concentration in milk together with advancing lactation is expected. Although the lactose concentration is known to stay relatively constant, changes nevertheless occur during the lactation: an increase at the beginning of lactation and a reduction in late lactation (Rook and Campling, 1965; Muir, 2002). However, citrate and lactose concentrations appear to have high biological variability between animals, making the change statistically less significant than the change in phosphorylated saccharides (Figure 5). Noteworthy is the low variability in the case of phosphosaccharides. For a parallel study, body energy balance was tracked in the same animals. Their period of negative energy balance varied from 19 to 72 DIM, with nadirs from −61 to −207 MJ on DIM 10 to 24 (Table 2). Therefore,

the hypothesis that energy balance could be the main driver of the decrease in phosphorylated saccharides in milk is unlikely.

As expected from the PCA results and high interindividual differences, changes in blood over 2 mo were less significant than those in milk. Signals m/z 520 to 521, 542 to 548, 61 (positive ions; Figure 3B), and 282 (negative ion) were those with at least a 50% increase, with $P < 0.001$. The positively charged ions at m/z 520 to 521 and 542 to 548 had fragments characteristic of phosphatidylcholines. From the molecular mass, the most probable FA residues in these lipids are linoleic acid and 20-carbon long-chain FA with 2 to 5 double bonds. Figure 6 presents the fragmentation spectra of m/z 522, which was strongly correlated with the listed signals and displayed fragmentation similar to the 2-oleoyl-3- phosphatidylcholine standard. A decrease in the PUFA concentration in milk during lactation has been reported before (Bitman and Wood, 1990; Garnsworthy et al., 2006; Gross et al., 2011). In blood, the reported changes have been similar to those in the current results. Increases have been observed in linoleic acid, the major FA in plasma lipids, within 30 or 60 DIM in the plasma lipid (Douglas et al., 2007; Contreras et al., 2010) and phospholipid fraction (Contreras et al., 2010). For arachidonic acid, stable values (Contreras et al., 2010) or a steady increase within 60 DIM in plasma lipids have been reported (Douglas et al., 2007). A postpartum decrease in plasma phospholipids after a steady increase up to 4 (van den Top et al., 1995) or 7 wk (Guretzky et al., 2006) postpartum has also been observed. Because blood phosphatidylcholine

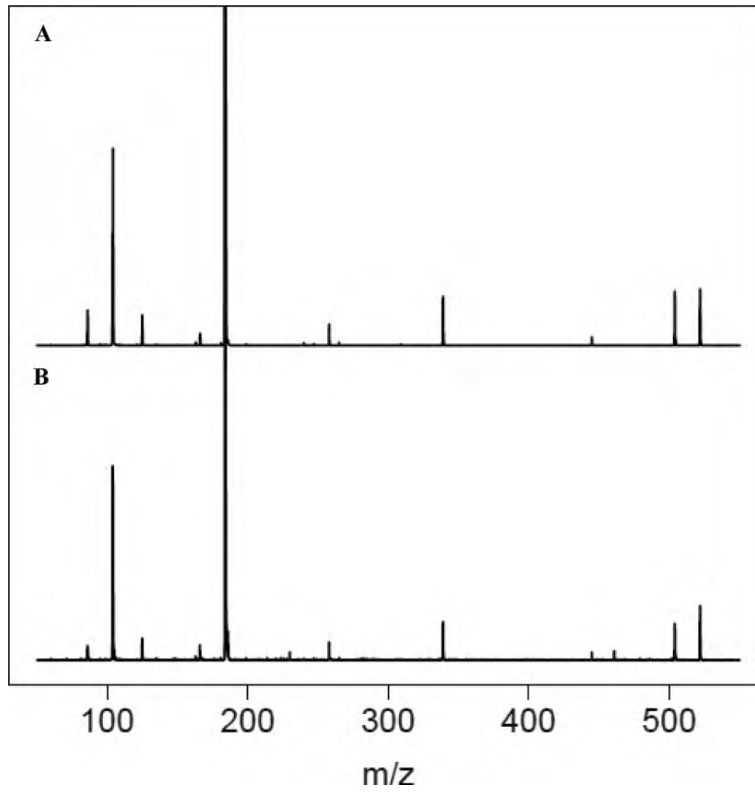


Figure 6. Fragmentation spectra of mass-to-charge ratio (m/z) 522 from a solution of commercial 2-oleoyl-3-phosphatidylcholine standard (A) and from plasma samples (B). The fragment at m/z 185 is plotted with 50% of its true intensity to bring the other signals into scale.

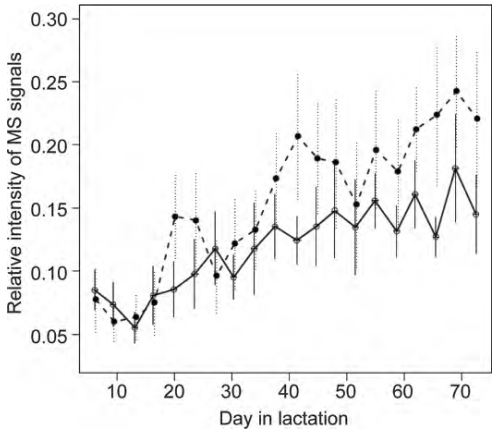


Figure 7. Levels of arachidonyl-phosphatidylcholine (solid line) and linoleyl-phosphatidylcholine (dashed line) in blood plasma during the first months of lactation. MS = mass spectrometry.

PUFA are used for the synthesis of milk fat (Easter et al., 1971), changes observed during the study period in blood phosphatidylcholine (an increase; Figure 7) coincide with the changes observed in milk citrate (a decrease) and are the result of the shift in the balance of preformed and de novo-synthesized FA in milk lipids: in the course of lactation, the proportion of preformed FA derived from blood decreases, and the proportion of de novo-synthesized FA increases (Kay et al., 2005)

The blood-to-milk correlation for the signals of PUFA-related molecules was weak, but this was not surprising considering the pathways of preformed milk FA and lipid synthesis in the mammary gland (Easter et al., 1971; Bauman and Griinari, 2003; Bauman et al., 2011).

Within the study period, the molecular composition of plasma was a result less of lactation-driven changes than of the composition of the milk. Additionally, plasma samples showed a higher degree of individuality, whereas the milk spectra from different animals were more readily comparable. Correlations between plasma and milk mass spectra were calculated. Only animal A showed a mean correlation (r = 0.2) between negative ion spectra from milk and plasma. All other comparisons gave means of $-0.1 < r < 0.1$. Therefore, the global metabolome, as a sum of individual compounds of milk and plasma, were not correlated. Nevertheless, a few individual compounds showed at least moderate correlations: most important, in the positive ionization mode m/z 114 (r = 0.47), identified as creatinine, and in the negative ionization mode m/z 184 (r = 0.40), 201 (r = 0.53), and 798 (r = 0.40). Fragmentation analysis indicated the presence of a carboxyl group in m/z 184 and a phosphate group in m/z 201, yet both compounds eluted late from the C18 column, implying the presence of hydrophobic moieties. It was surprising that m/z 301 (r = −0.41), 391 (r = −0.43), 554 to 557 (r = −0.5), and 616 (r = −0.47) were negatively correlated between blood and milk. Whether these negative correlations could be of biological importance or are false positive hits is yet unclear.

CONCLUSIONS

A study following changes in low molecular weight metabolites in milk and blood during the first months of lactation was performed. In the milk of different cows, common evolutions in time resolved. Citrate and lactose were found to contribute the most in apparent evolution over time, probably because of their abundance. Statistically, however, the most significant changes were attributed to phosphorylated saccharides, the levels of which decreased during the first 40 DIM. Little correlation was found between blood and milk metabolites; unsupervised classification of blood metabolomes was found to be based on the classes of individual cows. However, in blood a common increase was found in PUFA esters incorporated into phosphatidylcholine. This work demonstrates how minor metabolites may be significantly altered over the course of lactation and that a global metabolomic approach is a promising tool for finding such metabolites.

ACKNOWLEDGMENTS

This research was supported by grants from the Estonian Science Foundation (Tallinn; grants 7494 and 7856) and the Estonian Ministry of Education and Research (Tartu; SF 0170165s08). The publication of this study is supported by the Graduate School in Biomedicine and Biotechnology (Tartu; project no. 1.2.0401.09-0073).

REFERENCES

AOAC Int. 2005. Animal feed. Pages 1–72 in Official Methods of Analysis of AOAC International. 18th ed. W. Horwitz and G. W. Latimer, ed. AOAC Int., Gaithersburg, MD.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23:203–227.

Bauman, D. E., K. J. Harvatine, and A. L. Lock. 2011. Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. Annu. Rev. Nutr. 31:299–319.

Bitman, J., and D. L. Wood. 1990. Changes in milk fat phospholipids during lactation. J. Dairy Sci. 73:1208–1216.

Bossaert, P., J. L. M. R. Leroy, S. De Vliegher, and G. Opsomer. 2008. Interrelations between glucose-induced insulin response, metabolic

indicators, and time of first ovulation in high-yielding dairy cows. J. Dairy Sci. 91:3363–3371.
Boudonck, K., M. Mitchell, J. Wulff, and J. Ryals. 2009. Characterization of the biochemical variability of bovine milk using metabolomics. Metabolomics 5:375–386.
Chadeau-Hyam, M., T. M. D. Ebbels, I. J. Brown, Q. Chan, J. Stamler, C. C. Huang, M. L. Daviglus, H. Ueshima, L. Zhao, E. Holmes, J. K. Nicholson, P. Elliott, and M. De Iorio. 2010. Metabolic profiling and the metabolome-wide association study: Significance level for biomarker identification. J. Proteome Res. 9:4620–4627.
Contreras, G. A., N. J. O'Boyle, T. H. Herdt, and L. M. Sordillo. 2010. Lipomobilization in periparturient dairy cows influences the composition of plasma nonesterified fatty acids and leukocyte phospholipid fatty acids. J. Dairy Sci. 93:2508–2516.
DePeters, E. J., J. F. Medrano, and B. A. Reed. 1995. Fatty-acid composition of milk fat from three breeds of dairy cattle. Can. J. Anim. Sci. 75:267–269.
Douglas, G. N., J. Rehage, A. D. Beaulieu, A. O. Bahaa, and J. K. Drackley. 2007. Prepartum nutrition alters fatty acid composition in plasma, adipose tissue, and liver lipids of periparturient dairy cows. J. Dairy Sci. 90:2941–2959.
Easter, D. J., S. Patton, and R. D. McCarthy. 1971. Metabolism of phospholipid in mammary gland: I. The supply of phospholipid for milk synthesis in the rat and goat. Lipids 6:844–849.
Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 72:68–78.
Elgersma, A., G. Ellen, H. van der Horst, H. Boer, P. R. Dekker, and S. Tamminga. 2004. Quick changes in milk fat composition from cows after transition from fresh grass to a silage diet. Anim. Feed Sci. Technol. 117:13–27.
Fox, P. T. 2009. Milk: An overview. Pages 1–44 in Milk Proteins: From Expression to Food. A. Thompson, M. Boland, and H. Singh, ed. Elsevier Inc., Oxford, UK.
Garnsworthy, P. C., L. L. Masson, A. L. Lock, and T. T. Mottram. 2006. Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows. J. Dairy Sci. 89:1604–1612.
Grant, R. J., V. F. Colenbrander, and D. R. Mertens. 1990. Milk fat depression in dairy cows: Role of particle size of alfalfa hay. J. Dairy Sci. 73:1823–1833.
Gross, J., H. A. van Dorland, R. M. Bruckmaier, and F. J. Schwarz. 2011. Milk fatty acid profile related to energy balance in dairy cows. J. Dairy Res. 78:479–488.
Guretzky, N. A. J., D. B. Carlson, J. E. Garrett, and J. K. Drackley. 2006. Lipid metabolite profiles and milk production for Holstein and Jersey cows fed rumen-protected choline during the periparturient period. J. Dairy Sci. 89:188–200.
Harzia, H., K. Kilk, I. Jõudu, M. Henno, O. Kärt, and U. Soomets. 2012. Comparison of the metabolic profiles of noncoagulating and coagulating bovine milk. J. Dairy Sci. 95:533–540.
He, M., and L. E. Armentano. 2011. Effect of fatty acid profile in vegetable oils and antioxidant supplementation on dairy cattle performance and milk fat depression. J. Dairy Sci. 94:2481–2491.
Heuer, C., W. M. Van Straalen, Y. H. Schukken, A. Dirkzwager, and J. Noordhuizen. 2000. Prediction of energy balance in a high yielding dairy herd in early lactation: Model development and precision. Livest. Prod. Sci. 65:91–105.
Holtenius, K., S. Agenas, C. Delavaud, and Y. Chilliard. 2003. Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. J. Dairy Sci. 86:883–891.
Kay, J. K., W. J. Weber, C. E. Moore, D. E. Bauman, L. B. Hansen, H. Chester-Jones, B. A. Crooker, and L. H. Baumgard. 2005. Effects of week of lactation and genetic selection for milk yield on milk fatty acid composition in Holstein cows. J. Dairy Sci. 88:3886–3893.
Klein, M. S., M. F. Almstetter, G. Schlamberger, N. Nurnberger, K. Dettmer, P. J. Oefner, H. H. D. Meyer, S. Wiedemann, and W. Gronwald. 2010. Nuclear magnetic resonance and mass spectrometry-based milk metabolomics in dairy cows during early and late lactation. J. Dairy Sci. 93:1539–1550.
Klein, M. S., N. Buttchereit, S. P. Miemczyk, A.-K. Immervoll, C. Louis, S. Wiedemann, W. Junge, G. Thaller, P. J. Oefner, and W. Gronwald. 2012. NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. J. Proteome Res. 11:1373–1381.
McGeown, M. G., and F. H. Malpress. 1952. Studies on the synthesis of lactose by the mammary gland. II. The sugar-phosphate esters of milk. Biochem. J. 52:606–611.
Muir, D. D. 2002. Lactose: Properties, production, applications. Pages 1525–1529 in Encyclopedia of Dairy Sciences. Vol. 3. H. Roginski, J. W. Fuquay, and P. F. Fox, ed. Academic Press, London, UK.
Nielsen, N. I., K. L. Ingvartsen, and T. Larsen. 2003. Diurnal variation and the effect of feed restriction on plasma and milk metabolites in TMR-fed dairy cows. J. Vet. Med. A Physiol. Pathol. Clin. Med. 50:88–97.
Nousiainen, J., K. J. Shingfield, and P. Huhtanen. 2004. Evaluation of milk urea nitrogen as a diagnostic of protein feeding. J. Dairy Sci. 87:386–398.
Petzold, C. J., M. D. Leavell, and J. A. Leary. 2004. Screening and identification of acidic carbohydrates in bovine colostrum by using ion/molecule reactions and Fourier transform ion cyclotron resonance mass spectrometry: Specificity toward phosphorylated complexes. Anal. Chem. 76:203–210.
R Development Core Team. 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Accessed Jun. 12, 2012. http://www.R-project.org.
Rochfort, S. 2005. Metabolomics reviewed: A new "omics" platform technology for systems biology and implications for natural products research. J. Nat. Prod. 68:1813–1820.
Rook, J. A. F., and R. C. Campling. 1965. Effect of stage and number of lactation on the yield and composition of cow's milk. J. Dairy Res. 32:45–55.
Roux, A., D. Lison, C. Junot, and J. F. Heilier. 2011. Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: A review. Clin. Biochem. 44:119–135.
Sjaunja, L. O., L. Baevre, L. Junkkarinen, J. Pedersen, and J. Setala. 1990. A Nordic proposal for an energy corrected milk (ECM) formula. Pages 156–157 in Proc. Int. Comm. Anim. Recording (ICAR), 27th Session, Paris, France. EAAP Publication No. 50, 1991. Centre for Agricultural Publishing and Documentation (Pudoc), Wageningen, the Netherlands.
van den Top, A. M., T. Wensing, M. J. Geelen, G. H. Wentink, A. T. van't Klooster, and A. C. Beynen. 1995. Time trends of plasma lipids and enzymes synthesizing hepatic triacylglycerol during postpartum development of fatty liver in dairy cows. J. Dairy Sci. 78:2208–2220.
Waite, R., J. C. D. White, and A. Robertson. 1956. 613. Variations in the chemical composition of milk with particular reference to the solids-not-fat: I. The effect of stage of lactation, season of year and age of cow. J. Dairy Res. 23:65–81.
Zhang, A., H. Sun, P. Wang, Y. Han, and X. Wang. 2012. Recent and potential developments of biofluid analyses in metabolomics. J. Proteomics 75:1079–1088.

III

Harzia, H., Kilk, K., Ariko, T., Kass, M., Soomets, U., Jõudu, I., Kaart, T., Arney, D., Kärt, O., Ots, M. 2013
CRUDE GLYCEROL AS GLYCOGENIC PRECURSOR IN FEED; EFFECTS ON MILK COAGULATION PROPERTIES AND METABOLIC PROFILES OF DAIRY COWS
Journal of Dairy Research, 80 (2), 190-196

Journal of Dairy Research (2013) **80** 190–196. doi:10.1017/S0022029913000101

Crude glycerol as glycogenic precursor in feed; effects on milk coagulation properties and metabolic profiles of dairy cows

Hedi Harzia[1,2]*, Kalle Kilk[3], Tiia Ariko[1], Marko Kass[1,2], Ursel Soomets[3], Ivi Jõudu[1,2], Tanel Kaart[1,2], David Arney[1], Olav Kärt[1] and Meelis Ots[1,2]

[1] Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Kreutzwaldi 1, Tartu 51014, Estonia
[2] Bio-Competence Centre of Healthy Dairy Products, Kreutzwaldi 1, 51014 Tartu, Estonia
[3] Department of Biochemistry, Medical Faculty, University of Tartu, the Centre of Excellence for Translational Medicine, 50411 Tartu, Estonia

Received 16 March 2012; accepted for publication 20 December 2012; first published online 11 March 2013

As grain prices rise, the search for alternative glycogenic precursors in animal feed becomes increasingly important, and this study was conducted to determine if the replacement of starch with glycerol, as an alternative glycogenic precursor, affects the milk metabolic profile and milk coagulation ability, and therefore the quality of the milk. Eight primiparous mid-lactation Holstein cows were fed during a replicated 4 × 4 Latin square trial with four different isoenergetic rations: (1) control (T0) fed a total mixed ration (TMR) with barley meal; (2) group T1, decreased barley content, replaced isoenergetically with 1 kg crude glycerol; (3) group T2, the barley meal was replaced with 2 kg of crude glycerol; and (4) group T3 the barley meal was replaced with 3 kg of crude glycerol. Rumen, blood and milk samples were collected at the end of every 21-d treatment period. Rumen samples were analysed for proportion of total volatile fatty acid (VFA), blood samples for insulin and glucose, and milk for metabolites (e.g. citric-acid cycle compounds). The change in glycogenic precursors had a positive effect on rumen VFA proportions; the proportion of propionic acid increased ($P < 0{\cdot}001$). Milk protein ($P < 0{\cdot}001$) and curd firmness ($P < 0{\cdot}001$) both increased. The increase in milk protein concentration may have been due to an increase in microbial protein. Regarding the milk metabolic profiles, different signals were positively associated with coagulation ability and change in the diet. Based on this study, changing the glycogenic precursor in animal diet in this way is possible, and may have no immediate deleterious consequences on milk quality or cow health. Indeed, there is evidence for benefits from this substitution.

Keywords: Glycerol, glycogenic precursors, milk coagulation, metabolomics.

Introduction

Barley grain is a widely used animal feed in Northern Europe. It contains ~65% starch (Waldo, 1973), and is extensively (up to 90%) fermented in the rumen (Larsen et al. 2009), and the glycogenic precursor propionate is produced in abundance by starch fermentation. Rapidly degradable starch is a good energy source for rumen microbes, and it is fermented into volatile fatty acids (VFA), which are cows' major source of energy (Huntington, 1997).

VFA proportion in the rumen is associated with diet composition (Murphy et al. 1982), milk yield and its composition (Thomas et al. 1988). Of the VFA produced in the rumen, propionate, isobutyrate and valerate, are used for glucose production in the liver and are thus precursors for gluconeogenesis (Larsen et al. 2009). Most of the organic acids as well as the precursors for gluconeogenesis, adsorb through the rumen wall and are removed from blood by the liver to be synthesized into glucose. During lactation, over 70% of the synthesized glucose is used for milk production (Elliot, 1976), and is irreversibly lost to the animal with the milk. Therefore there is always requirement for new glucose, and a need for the diet to meet cows' metabolic requirements. This is particularly important for high-producing cows, especially during the transition period, when the energy output exceeds the input. To ensure energy balance during the transition period glycerol, as a sweet liquid substance and by-product of biodiesel production (Donkin & Doane, 2007), has been used as a feed additive as an additional glucose precursor (DeFrain et al. 2004). According to DeFrain et al. (2004) glycerol, fed as a part of a total mixed ration (TMR), is mainly used as an energy substrate by rumen microbes, rather than directly contributing to milk synthesis

*For correspondence; e-mail: hedi.harzia@emu.ee

via gluconeogenesis. Accordingly there should be no direct effect of glycerol feed on milk production and composition, although Khalili et al. (1997) and Donkin et al. (2009) have stated otherwise. In addition to the amount and composition of milk produced, milk coagulation ability, the basis of cheese making, is also an important trait. Different laboratories have studied the effect of diet on milk coagulation properties (Malossini et al. 1996; Guinee et al. 2001), and differences in metabolic profiles of raw milk with different coagulation ability have also been noted (Sundekilde et al. 2011; Harzia et al. 2012). But still, to date, there is a lack of studies describing the effects of changing the glycogenic precursors on milk coagulation properties and metabolic profile.

In this study, the effects of different amounts of reduced barley meal and fed crude glycerol on milk were studied. It was hypothesized that crude glycerol in the diet alters the profile of low molecular weight compounds in milk. The aim was to find out if a change in the glycogenic precursor alters the milk metabolic profile and technological properties (e.g. coagulation) of the milk.

Materials and methods

Cows and dietary treatment

Eight primiparous Estonian Holstein dairy cows (days in milk, DIM 134 ± 5) were used in a replicated 4 × 4 Latin square trial; one of each of the replicates was fitted with a ruminal fistula. Cows were divided into pairs according to milk yield (24·7 ± 1·0 kg/d) and body weight (535 ± 13·5 kg), and each treatment period lasted 21 d (16 d adaptation period and 5 d of data collection). During the experimental period the cows were fed with TMR twice a day, and milked before feeding. Based on Estonian feeding recommendations (Vabariiklik Söötmisalase Uurimistöö Koordineerimise Komisjon, 1995) the base ration contained grass silage, barley meal, soya meal, limestone, sodium chloride and lactating cow mineral mix (Table 1). Feed samples were analyses according to established methods (AOAC, 2005). Experimental diets consisted of the base diet, containing only barley meal and no crude glycerol (control; T0) or crude glycerol in the following amounts: 1 (T1), 2 (T2) or 3 kg (T3). Liquid crude glycerol with metabolisable energy value of 14 MJ/kg (Mach et al. 2009), was hand-mixed to TMR. The quantity of crude glycerol increased isoenergetically to the decrease in the quantity of barley meal. Optigen II was used to replace the difference in crude protein in experimental diets. The experiment was carried out according to the Estonian Animal Protection Act at the Eerika Experimental Farm of the Estonian University of Life Sciences (Märja, Estonia).

Table 1. Feed ingredients and chemical composition of experimental diet g/kg of DM during different treatments (T0, T1, T2 and T3)

Ingredients	T0	T1	T2	T3
Grass silage	469	470	471	472
Soybean meal	111	112	112	112
Barley meal	394	339	283	227
Crude glycerol†	0	52	104	156
Optigen II‡	0	3	6	8
Mineral mix§	11	11	11	11
Calcium carbonate	8	8	8	8
Sodium chloride	6	6	6	6
Chemical composition				
Organic matter	919	865	812	758
Crude protein	155	156	157	158
Neutral detergent fibre	363	351	339	327
Acid detergent fibre	221	217	213	209
Metabolizable energy (MJ)	11·1	11·2	11·2	11·2

† BioOil Ltd, Estonia (82·6% glycerol, 9·3% salts, 7·1% water, 0·6 ether extract and 0·4% methanol)

‡ Alltech, USA (41·0% nitrogen and 11·4% crude fat)

§ Veskimeister Ltd, Estonia (contained $CaCO_3$ 30%, NaCl 20%, $Ca(H_2PO_4)_2$ 20%, $Mg_3(PO_4)_2$ 19·5%; Fe – 2000 mg/kg, Zn – 6000 mg/kg, Mn – 3000 mg/kg, Cu – 750 mg/kg, Se – 30 mg/kg, I – 150 mg/kg, Co – 50 mg/kg, 450 000 IU/kg of vitamin A, 100 000 IU/kg of vitamin D, and 3000 IU/kg of vitamin E)

Data collection and analysis

Rumen liquid samples were taken at the end of each treatment period (d 20 and 21) to analyse VFA composition (propionic, valeric and isobutyric acids). Acid proportion was measured using the method described by (Cottyn & Boucque, 1968), and was analysed by an Agilent 7890A gas chromatograph (Agilent Technologies Inc, USA) using 4% Carbowax 20 M, matrix 80/120 Carbopack B-DA column (Sigma-Aldrich, St. Louis, USA).

Blood samples were collected from the coccygeal vein on the 20th and 21st experimental day using 10-ml heparin Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes NJ, USA), and analysed for insulin and glucose concentrations. Plasma samples for insulin concentrations were analysed radio-immunologically (Wallac 1470 Wizard Gamma Counter; Perkin Elmer Life and Analytical Sciences, Inc., USA) using ^{125}I radioimmunoassay test kits (Coat-A-Count Insulin, Siemens Medical Solutions Diagnostics, USA). Spectrophotometrical analysis (Helios β; Unicam Ltd., PO Box 206, York St., Cambridge, CB1 2ST, UK) of plasma glucose concentrations with Randox reagents (Ranbut, Randox Laboratories Ltd, United Kingdom) was performed.

Milk yield was recorded on the last 5 d of the experimental period, and samples were collected at the end of each treatment period (d 20 and 21). Milk samples were stabilised with bronopol (Broad Spectrum Microtabs, D & F Control Systems Inc., Norwood, USA). Concentrations of milk fat, protein, urea and somatic cell count (SCC) were measured in each milk sample using an automated infrared milk analyser (System 4000, Foss Electric, Hillerød, Denmark). Milk pH was measured using a pH-meter (MP 220; Mettler Toledo GmbH, Greifensee, Switzerland) before the coagulation

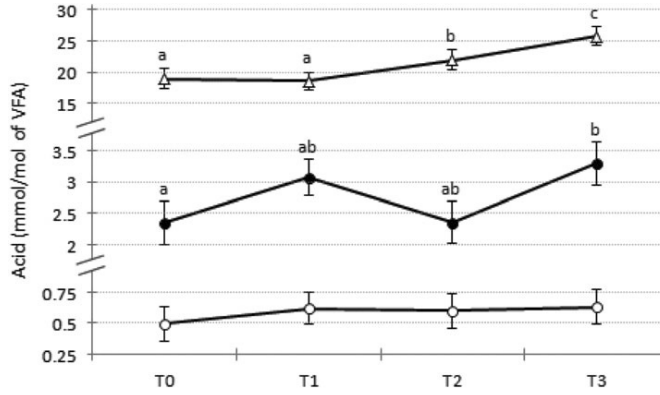


Fig. 1. Least square means (± standard error) of isobutyric (○), valeric (●) and propionic (Δ) acid depending on the diet. [abc] Least square means with different letters are statistically significantly different ($P<0{\cdot}05$).

ability was analysed, at 20 °C. Curd firmness (E_{30}) and coagulation time (RCT) were obtained by Optigraph (Alliance Instruments, France) using the method described previously by Kübarsepp et al. (2005).

The milk samples for mass spectrometry analyses were prepared and analysed as previously described by Harzia et al. (2012). The milk samples for mass spectrometry analyses were prepared and analysed as previously described by Harzia et al. (2012). For preliminary citric-acid cycle compound quantification HILIC (Luna 5 µM HILIC 200A, 150 × 3·00 mm, Phenomenox, Torrance, USA) column and standard solutions were used. The gradient for retention time studies was as follows: 5 min isocratic at 95% ammoniumformate in methanol, gradual decline to 5% ammoniumformate in methanol within 15 min, 5 min at 5% acetonitrile in water. Negative ions formed in the TurboIonSpray source were scanned in the Multiple Reaction Monitoring mode (MRM). The scan rate was 1000 amu/s. Curtain and nebulizer gas had settings at 10 and 5, respectively. Ionization was performed at a temperature of 200 °C. The Ion Spray voltage was set at 4500 V. The entrance and declustering potential, and the collision energy, were set at 20, 10 and 2 V, respectively.

Statistical analysis

The statistical significance of treatment effect was tested following the model $y_{ijkl}=\mu+D_i+P_j+C_k+e_{ijkl}$, where y_{ijkl} is the dependent variable, μ is the model intercept, D_i is the diet effect ($i=1,\ldots,4$), P_j is the period effect ($j=1,\ldots,4$), C_k is the random cow effect ($k=1,\ldots,8$), and e_{ijkl} is the model error. Single treatments were compared according to their least square means. To study the associations between different VFA, blood and milk metabolites, and milk metabolic profile, Spearman partial correlation coefficients, adjusted for the period effect, were estimated. All statistical analyses were performed with SAS software (version 9.1; SAS Institute Inc., Cary, NC, USA).

Results and discussion

Effect of glucogenic precursors on rumen VFA and milk composition

A change in the proportions of glycogenic VFAs was observed when starch supplementation was partially replaced with crude glycerol (Fig. 1). The proportion of propionic acid produced changed as an effect of treatment, being significantly different from the control at T2 and T3 ($P=0{\cdot}03$, and $P<0{\cdot}001$, respectively). A strong positive correlation was observed between propionic acid and glycerol addition ($r=0{\cdot}71$, $P=0{\cdot}0002$), and the change in the valeric acid proportion was significantly different between the control and T3 ($P=0{\cdot}03$).

As stated previously, the proportion of propionic acid increased during this study. According to Huhtanen et al. (1993) the milk yield is limited by propionate. This was not the case in the current study. Huhtanen et al. (1993) also noted that the use of propionate for gluconeogenesis in the liver may be inhibited by butyrate, leading to decreased glucose production, and increasing the utilization of amino acids for gluconeogenesis, thereby reducing the availability of amino acids (AA) to the mammary gland. In the current study the proportion of butyrate produced changed ($P<0{\cdot}001$) in the same direction as propionic acid, indicating that the equilibrium between these two acids remained during the whole treatment diet, and change in AA metabolism should not have been affected. According to Ørskov (1986) there can be problems utilizing a large volume of propionate; as the concentration of propionate

increases in blood, insulin production also increases. When insulin production is stimulated, the uptake of glucose in different tissues is increased and lipolysis is reduced, and milk production and milk fat is decreased (Ørskov, 1986; Hayirli, 2006). The change in the proportion of propionate produced indicated that glucose availability, through gluconeogenesis in liver and in blood, should have increased. Contrary to Wang et al. (2009), who observed an increase in plasma glucose when feeding glycerol, in this study there was no significant increase in either glucose (from 85·97 to 88·47 mg/dl, $P=0·5$) or insulin concentrations (from 14·13 to 14·71 μIU/ml, $P=0·8$). The changes in blood metabolites were not significant nor had a definite trend curve with increasing glycerol in the diet. But a strong negative correlation between insulin level and milk yield was observed ($r=-0·40$, $P=0·003$). The change in insulin concentration was probably too small to have an affect on either liver or peripheral tissues, as there was no change in BW, this being consistent with results reported by Boyd et al. (2011).

Overall, variations between different milk traits were not significantly different, except for protein concentration between all treatments ($P<0·001$), and for pH between the control and T3 ($P=0·01$). The increase in milk protein concentration may have been due to a faster outflow of microbial protein from the rumen (Pathak, 2008), thus increasing amino acid production, which may be converted into milk protein. A significant change in lactose concentration was observed between the control and T1 ($P=0·03$), and also between T1 and T3 ($P=0·02$). Circulating glucose, which is taken up by the udder, is used to synthesize lactose, and to provide enough energy for fat synthesis (Rigout et al. 2003). The blood glucose level had weak correlation with milk lactose concentration ($r=0·18$, $P=0·19$), and was negatively correlated with milk fat ($r=-0·36$, $P=0·01$). A negative correlation was also observed between milk lactose and fat concentrations ($r=-0·34$, $P=0·01$), indicating that the glucose taken up by the udder ensured a sustainable level for lactose synthesis, as fat synthesis depends on glucose availability and lactose synthesis.

Effect of glycogenic precursors on milk coagulation properties and metabolites

Least square mean values of milk yield, somatic cell score (SCS; SCS = ln (SCC)), fat, urea, lactose and protein concentration, pH, RCT and E_{30}, of all four rations are shown in Table 2.

Mean milk coagulation ability, measured as $E_{30,}$ improved linearly as the barley concentration decreased and glycerol supplementation increased ($P<0·001$). According to Jõudu et al. (2008) protein concentration has a positive effect on milk coagulation ability. In the current study there was a strong positive correlation between curd firmness and protein concentration ($r=0·58$, $P<0·001$).

There was a negative correlation between isobutyric acid and curd firmness ($r=-0·56$, $P=0·005$), and a trend for a positive correlation between propionic acid and curd firmness ($r=0·38$ and $P=0·07$), but none for valeric acid ($r=0·32$ and $P=0·13$). These findings indicate that a change in rumen VFA proportion is related to milk coagulation ability, which may be a result of a change in the microbial population during the period of the experimental diet.

Table 2. Least square means of concentrations of different milk traits and milk metabolites with different treatments (T0, T1, T2, and T3). Values are least square means for $n=64$. Standard errors of mean of all least square mean values are given as SEM

Trait†	T0	T1	T2	T3	SEM
Milk yield, kg/d	22·5	22·8	23·0	22·5	0·82
Fat, g/100 g	4·61	4·64	4·60	4·54	0·12
Protein, g/100 g	$3·62^{a}$	$3·69^{b}$	$3·72^{b}$	$3·75^{b}$	0·08
Urea, mg/l	221	220	234	228	7·16
Lactose, g/100 g	$4·79^{a}$	$4·75^{b}$	$4·79^{ab}$	$4·80^{a}$	0·04
pH	$6·64^{ab}$	$6·65^{a}$	$6·64^{ab}$	$6·66^{b}$	0·01
SCS	3·83	3·73	3·97	3·78	0·20
E_{30}, mm	$28·4^{a}$	$30·9^{a}$	$31·9^{a}$	$35·9^{b}$	2·58
RCT	9·10	9·10	9·05	9·08	0·15
Pyruvic acid, μM	0·22	0·22	0·22	0·22	0·02
Citric acid, μM	21·6	16·1	19·8	23·2	5·20
Cis-aconitic acid, μM	3·69	3·90	3·83	4·11	0·68
α-Ketoglutaric acid, μM	7·04	7·70	7·78	7·81	1·58
Malonic acid, μM	7·64	8·28	8·64	8·29	1·59
Oxaloacetic acid, μM	6·11	7·30	5·85	11·3	2·72

[a, b, c] Means within a row with different superscripts are statistically different ($P<0·05$)

†SCS – ln(somatic cell count). E_{30} – curd firmness after 30 min. RCT – coagulation time

The hypothesis of this study was that the change of glycogenic precursor alters the milk metabolic profile. Therefore mass spectrometry analysis and identification of low molecular weight milk compounds was carried out to gain more knowledge about reasons behind the change in coagulation ability. A change in the metabolic profile for milk samples with different curd firmness was observed with treatment. In the positive ion mode there were 20 statistically different signals representing differences between coagulation ability, and 17 of these were negatively correlated with coagulation. In addition, mass to charge ratios (m/z) describing relationships between coagulation ability and change in the glycogenic precursor in the diet were investigated (Fig. 2). Signals located in the upper right corners of Fig. 2A, B were positively correlated with both glycerol addition and curd firmness, indicating that the higher amount of crude glycerol corresponded to the stronger mass spectrometry signals of these masses, which is further reflected in the higher values for curd firmness. From the recorded signals, only one was significantly different for both variables. Signals $m/z=293$ represented good coagulation and glycerol addition to the diet (Fig. 2A). Fragment analysis was conducted on this signal, and a database search resulted in the finding that it had fragments identical to histidine, His–His and lysine as the $[2M-H]^{+}$ ion. The abundance of both of these amino acids in the well coagulating milk

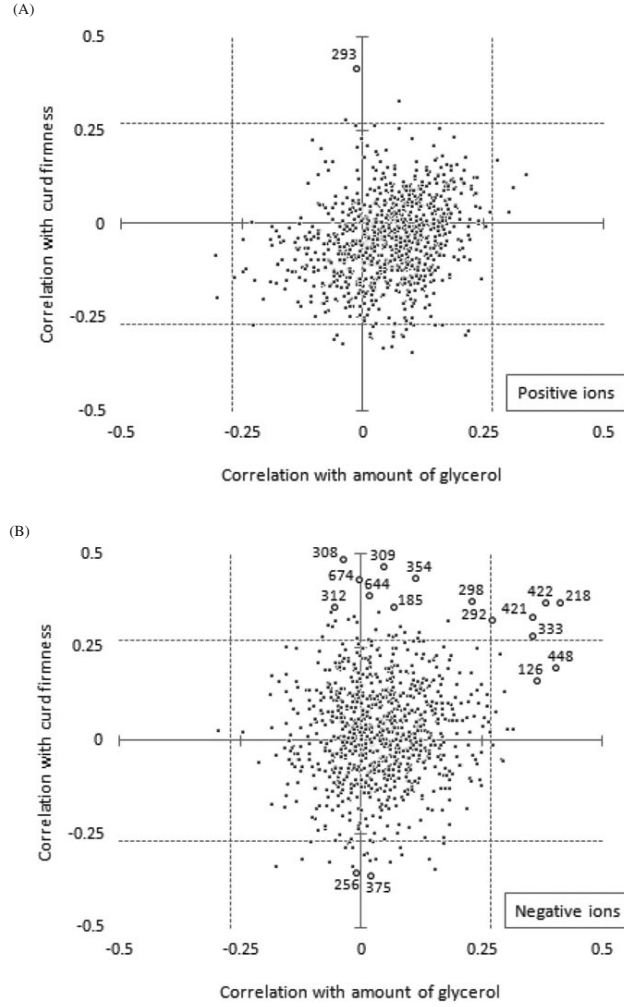


Fig. 2. Spearman partial correlations (adjusted for period effect) of masses measured in positive ion mode (A) and negative ion mode (B) with glycerol addition and curd firmness. Larger labelled dots correspond to correlations with $P<0{\cdot}01$ in a horizontal or a vertical direction or indicate masses significantly ($P<0{\cdot}05$) correlated with both glycerol addition and curd firmness. Dashed lines denote the cut-off for statistical significance of correlation coefficients ($P=0{\cdot}05$).

samples is explained by increased protein concentrations. Histidine, and lysine, and also methionine are the limiting amino acids for milk protein production (Kim et al. 2001).

In negative ion mode the number of statistically different ($P<0{\cdot}05$) signals was higher (53), and five of these represented both coagulation and change in glycogenic precursor in the diet (Fig. 2B). These five correlations corresponded to the signals $m/z=218$, 422, 421, 333 and 292, and fragmentation analyses was also carried out. No defined matches were found for the signals $m/z=421$, 422,

and 333. Although the fragment spectra of signal $m/z = 421$ had fragments common to cortisol, such as the $[M\text{-}H]^-$ ion, and signal $m/z = 333$ had a fragment common to decanoic acid $[M\text{-}H]^-$. Signal $m/z = 292$ had fragment spectra matching glycerophosphocholine. According to Klein et al. (2012) the ratio of glycerophosphocholine to phosphocholine is a good biomarker for ketosis, where high values of glycerophosphocholine indicate a low risk of ketosis. Fragment spectra of signal $m/z = 218$ indicated pantothenic acid, the $[M\text{-}H]^-$ ion. Ruminants either obtain their pantothenic acid from feed, or it is synthesized by the rumen microbes (Bechdel et al. 1928; Bender, 2009). The occurrence of free pantothenic acid in milk is rare; usually it is bound to Coenzyme A, and therefore involved in energy metabolism in cells (Bender, 2009). According to previous studies (Lardinois et al. 1944; Hollis et al. 1954; Hayes et al. 1967) the synthesis of pantothenic acid depends on the diet, and increases as the supplementation of rapidly degradable carbohydrates is increased. Although Bonomi (2000) observed that pantothenic acid has no effect on milk coagulation, it was found in the current study that it was positively correlated with $E_{30,}$ and also with a change in the glycogenic precursor in the diet.

To analyse impacts of changes in the diet on the milk energy profile, milk organic acid composition was analysed, and changes in citric-acid cycle components' (e.g. citrate, pyruvate, cis-aconitate, α-ketoglutarate, malonate, and oxaloacetate; Table 2) concentrations between treatment periods were measured. Of the organic acids in milk, oxaloacetic acid was positively correlated with curd firmness ($r = 0{\cdot}33$, $P = 0{\cdot}013$) while there was a trend for malonic acid and curd firmness ($r = 0{\cdot}25$, $P = 0{\cdot}06$). No correlations were observed between citrate and cis-aconitate with curd firmness ($r = -0{\cdot}01$, $P = 0{\cdot}94$, and $r = 0{\cdot}02$, $P = 0{\cdot}88$, respectively). No correlations were found between curd firmness and pyruvic, and α-ketoglutaric acid ($r = 0{\cdot}13$, $P = 0{\cdot}34$, and $r = 0{\cdot}11$, $P = 0{\cdot}42$, respectively).

As the main buffer system of milk, citrate is a common milk component affecting milk-processing quality (e.g. coagulation) by interacting with other milk constituents (Rosenthal, 1991), as the citrate ions can improve calcium and phosphate ions binding to casein micelles (Visser et al. 1979). As an intermediate in the citric-acid cycle, citric acid takes a role in cellular energy metabolism, and therefore it has been reported that citric acid is an indicator of energy status in the cow (Baticz et al. 2002). Linzell et al. (1976) indicated that mammary epithelium is impermeable to citrate, it is formed in mammary secretory cells and its abundance in milk depends on season (Holt & Muir, 1979; Mitchel, 1979; Keogh et al. 1982), lactation (Garnsworthy et al. 2006), and can be altered by feeding (Faulkner & Peaker, 1982). However the data in the literature about the effect of feeding are inconsistent. As mentioned by Gransworthy et al. (2006), bovine milk citrate concentration is not affected by either milk yield or diet. In the current study the concentrations of milk citric-acid cycle components changed insignificantly with treatment.

In conclusion, this study has shown that a change in the glycogenic precursor in the diet can alter the milk metabolic profile, and may improve its coagulation ability through altered proportions of rumen VFA, milk protein and concentrations of energy metabolites (e.g. citric-acid cycle components, pantothenic acid) in milk. As was hypothesized, the crude glycerol in the diet alters the profile of low molecular weight compounds, and technological properties (e.g. coagulation) of the milk. Therefore using glycerol as a dietary source for glucogenesis appears to be of potential value in the feeding of the dairy cow.

This research was carried out by the Bio-Competence Centre of Healthy Dairy Products and was co-financed by the European Community's Regional Development Fund within the framework of the Competence Centre Programme of the Enterprise Estonia, project no. EU30002, targeted finance project SF0170165s08, and Estonian Science Foundation grants 7494 and 7856.

References

Association of Official Analytical Chemists (AOAC) 2005 *Official Methods of Analysis of AOAC International*, 18th Edition. Gaithersburg, MD, USA: AOAC International

Baticz O, Tomoskozi S, Vida L & Gaal T 2002 Relationship between concentration of citrate and ketone bodies in cow's milk. *Acta Veterinaria Hungarica* **50** 253–261

Bechdel SI, Honeywell HE, Dutcher RA & Knutsen MH 1928 Synthesis of vitamin B in the rumen of the cow. *Journal of Biological Chemistry* **80** 231–238

Bender DA 2009 Pantothenic acid. In *Nutritional Biochemistry of the Vitamins*, pp. 341–359 (Ed. DA Bender). Cambridge: Cambridge University Press

Bonomi A 2000 Dairy cattle ration integration with rumen-protected pantothenic acid. Effects on milk production and reproductive efficiency. *Rivista di Scienza dell'Alimentazione* **29** 321–338

Boyd J, West JW & Bernard JK 2011 Effects of the addition of direct-fed microbials and glycerol to the diet of lactating dairy cows on milk yield and apparent efficiency of yield. *Journal of Dairy Science* **94** 4616–4622

Cottyn BG & Boucque CV 1968 Rapid method for the gas-chromatographic determination of volatile fatty acids in rumen fluid. *Journal of Agricultural and Food Chemistry* **16** 105–107

DeFrain JM, Hippen AR, Kalscheur KF & Jardon PW 2004 Feeding glycerol to transition dairy cows: effects on blood metabolites and lactation performance. *Journal of Dairy Science* **87** 4195–4206

Donkin SS & Doane P 2007 Glycerol as a feed ingredient in dairy rations. In *Proceedings of the Tri-State Dairy Nutrition Conference, Proceedings*, pp. 97–103 Fort Wayne, IN: Ohio State University, Columbus

Donkin SS, Koser SL, White HM, Doane PH & Cecava MJ 2009 Feeding value of glycerol as a replacement for corn grain in rations fed to lactating dairy cows. *Journal of Dairy Science* **92** 5111–5119

Elliot JM 1976 The glucose economy of the lactating dairy cow. In *Proceedings of the Cornell Nutrition Conference for Feed Manufacturors*, pp. 59–66 Ithaca, NY: Cornell University

Faulkner A & Peaker M 1982 Reviews of the progress of dairy science: secretion of citrate into milk. *Journal of Dairy Research* **49** 159–169

Garnsworthy PC, Masson LL, Lock AL & Mottram TT 2006 Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows. *Journal of Dairy Science* **89** 1604–1612

Guinee TP, Mulholland EO, O'Brien B & Murphy JJ 2001 Effect of diet quality on the suitability of mid-lactation bovine milk for cheddar cheese manufacture. *Australian Journal of Dairy Technology* **56** 3–8

Harzia H, Kilk K, Jõudu I, Henno M, Kärt O & Soomets U 2012 Comparison of the metabolic profiles of noncoagulating and coagulating bovine milk. *Journal of Dairy Science* **95** 533–540

Hayes BW, Mitchell GE Jr. & Little CO 1967 Factors affecting concentrations of B-vitamins in ruminal fluid in vitro. *Internationale Zeitschrift für Vitaminforschung* **37** 348–353

Hayirli A 2006 The role of exogenous insulin in the complex of hepatic lipidosis and ketosis associated with insulin resistance phenomenon in postpartum dairy cattle. *Veterinary Research Communications* **30** 749–774

Hollis L, Chappel CF, MacVicar R & Whitehair CK 1954 Effect of ration on vitamin synthesis in rumen of sheep. *Journal of Animal Science* **13** 732–738

Holt C & Muir DD 1979 Inorganic constituents of milk: I. Correlation of soluble calcium with citrate in bovine milk. *Journal of Dairy Research* **46** 433–439

Huhtanen P, Miettinen H & Ylinen M 1993 Effect of increasing ruminal butyrate on milk yield and blood constituents in dairy cows fed a grass silage-based diet. *Journal of Dairy Science* **76** 1114–1124

Huntington GB 1997 Starch utilization by ruminants: from basics to the bunk. *Journal of Animal Science* **75** 852–867

Jõudu I, Henno M, Kaart T, Püssa T & Kärt O 2008 The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows. *International Dairy Journal* **18** 964–967

Keogh MK, Kelly PM, O'Keeffe AM & Phelan JA 1982 Studies of milk composition and its relationship to some processing criteria: II. Seasonal variation in the mineral levels of milk. *Irish Journal of Food Science and Technology* **6** 13–27

Khalili H, Varvikko T, Toivonen V, Hissa K & Suvitie M 1997 The effects of added glycerol or unprotected free fatty acids or a combination of the two on silage intake, milk production, rumen fermentation and diet digestibility in cows given grass silage based diets. *Agricultural and Food Science in Finland* **6** 349–362

Kim C-H, Choung J-J & Chamberlain DG 2001 Estimates of the efficiency of transfer of L-histidine from blood to milk when it is the first-limiting amino acid for secretion of milk protein in the dairy cow. *Journal of the Science of Food and Agriculture* **81** 1150–1155

Klein MS, Buttchereit N, Miemczyk SP, Immervoll AK, Louis C, Wiedemann S, Junge W, Thaller G, Oefner PJ & Gronwald W 2012 NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. *Journal of Proteome Research* **11** 1373–1381

Kübarsepp I, Henno M, Kärt O & Tupasela T 2005 A comparison of the methods for determination of the rennet coagulation properties of milk. *Acta Agriculturae Scandinavica Section a – Animal Science* **55** 145–148

Lardinois CC, Mills RC, Elvehjem CA & Hart EB 1944 Rumen Synthesis of the Vitamin B Complex as Influenced by Ration Composition. *Journal of Dairy Science* **27** 579–583

Larsen M, Lund P, Weisbjerg MR & Hvelplund T 2009 Digestion site of starch from cereals and legumes in lactating dairy cows. *Animal Feed Science and Technology* **153** 236–248

Linzell JL, Mepham TB & Peaker M 1976 The secretion of citrate into milk. *Journal of Physiology* **260** 739–750

Mach N, Bach A & Devant M 2009 Effects of crude glycerin supplementation on performance and meat quality of Holstein bulls fed high-concentrate diets. *Journal of Animal Science* **87** 632–638

Malossini F, Bovolenta S, Piras C, DallaRosa M & Ventura W 1996 Effect of diet and breed on milk composition and rennet coagulation properties. *Annales De Zootechnie* **45** 29–40

Mitchel LGE 1979 Seasonal variation in citrate content of milk. *Australian Journal of Dairy Technology* **34** 158–160

Murphy MR, Baldwin RL & Koong LJ 1982 Estimation of stoichiometric parameters for rumen fermentation of roughage and concentrate diets. *Journal of Animal Science* **55** 411–421

Ørskov ER 1986 Starch digestion and utilization in ruminants. *Journal of Animal Science* **63** 1624–1633

Pathak AK 2008 Various factors affecting microbial protein synthesis in the rumen. *Veterinary World* **1** 186–189

Rigout S, Hurtaud C, Lemosquet S, Bach A & Rulquin H 2003 Lactational effect of propionic acid and duodenal glucose in cows. *Journal of Dairy Science* **86** 243–253

Rosenthal I 1991 *Milk and Dairy Products: Properties and Processing*. New York: VHC Publisher

Sundekilde UK, Frederiksen PD, Clausen MR, Larsen LB & Bertram HC 2011 Relationship between the metabolite profile and technological properties of bovine milk from two dairy breeds elucidated by NMR-based metabolomics. *Journal of Agricultural and Food Chemistry* **59** 7360–7367

Thomas GB, Pearce DT, Oldham CM, Martin GB & Lindsay DR 1988 Effects of breed, ovarian steroids and season on the pulsatile secretion of LH in ovariectomized ewes. *Journal of Reproduction and Fertility* **84** 313–324

Waldo DR 1973 Extent and partition of cereal grain starch digestion in ruminants. *Journal of Animal Science* **37** 1062–1074

Wang C, Liu Q, Yang WZ, Huo WJ, Dong KH, Huang YX, Yang XM & He DC 2009 Effects of glycerol on lactation performance, energy balance and metabolites in early lactation Holstein dairy cows. *Animal Feed Science and Technology* **151** 12–20

Vabariiklik Söötmisalase Uurimistöö Koordineerimise Komisjon 1995 *Põllumajandusloomade söötmisnormid koos söötade tabelitega* (Feeding Recommendations and Nutrient tables for Farm Animals) (Eds Ü Oll & S Tölp). Tartu: Eesti Põllumajandusülikool

Visser J, Schaier RW & van Gorkom M 1979 The role of calcium, phosphate and citrate ions in the stabilization of casein micelles. *Journal of Dairy Research* **46** 333–335

IV

Harzia, H., Ilves, A., Ots, M., Henno, M., Jõudu, I., Kaart, T., Ling, K., Kärt, O., Kilk, K., Soomets, U. 2013
ALTERATIONS IN MILK METABOLOME AND COAGULATION ABILITY DURING THE LACTATION OF DAIRY COWS
Journal of Dairy Science, 96 (10), 6440-6448

J. Dairy Sci. 96:6440–6448
http://dx.doi.org/10.3168/jds.2013-6808

Alterations in milk metabolome and coagulation ability during the lactation of dairy cows

H. Harzia,*†‡[1] A. Ilves,*†‡ M. Ots,*† M. Henno,* I. Jõudu,*† T. Kaart,*† K. Ling,* O. Kärt,* K. Kilk,‡ and U. Soomets‡
*Institute of Veterinary Medicine and Animal Science, Estonian University of Life Sciences, Kreutzwaldi 1, Tartu 51014, Estonia
†Bio-Competence Centre of Healthy Dairy Products, Kreutzwaldi 1, 51014 Tartu, Estonia
‡Department of Biochemistry, Medical Faculty, University of Tartu, the Centre of Excellence for Translational Medicine, 50411 Tartu, Estonia

ABSTRACT

Milk composition has been known to change during lactation. To help understand the changes in metabolic profile throughout the whole lactation, liquid chromatography mass-spectrometry was used to analyze 306 milk samples from 82 primi- and multiparous dairy cows. Changes in metabolic profile common to all cows throughout lactation were ascertained based on principal component and general linear model analysis. Sets of specific markers; for instance, 225, 397, and 641–642 m/z (positive mode), and 186, 241, and 601–604 (negative mode), with at least a 1.5-fold higher intensity during the first 60 d compared with the last 60 d of lactation were observed. The metabolome was affected by parity and milking time. Markers, identified as peptides differentiating parity, were observed. A significant increase for citrate was observed in evening milk. Milk coagulation traits were strongly animal specific. The curd firmness values were influenced by milking time. Sets of markers were associated with curd firmness in positive (197 m/z) and negative (612, 737, 835, 836, 902, 1000, 1038, and 1079 m/z) ion mode.
Key words: lactation curve, milk metabolite, coagulation

INTRODUCTION

Metabolomics has been introduced recently to animal and dairy science. Being routinely collected, milk is a suitable substance for monitoring analyses. A better understanding of the milk metabolome would advance its use in evaluating the state of the animals and milk technological properties. Milk composition and production are affected by inherent and external factors, directly or indirectly. Differences in milk composition may be caused by nutritional (Malossini et al., 1996) or nonnutritional factors such as stage of lactation (Jõudu et al., 2008; Stoop et al., 2009). Using nuclear magnetic resonance (Klein et al., 2010, 2012) and GC-MS (Klein et al., 2010) for targeted analyses, a change in the metabolic composition of milk throughout the lactation has been observed. In a recent study by Ilves et al. (2012), using a mass spectrometric approach (liquid chromatography-tandem MS; **LC-MS/MS**), changes in the milk metabolome in early lactation were observed, specifically decreases in phosphorylated saccharides, citrate, and lactose concentrations. Melzer et al. (2013) also showed changes in the metabolome up to d 120 of lactation; metabolites that correlated with milk traits were detected. However, one limitation of the study was the lack of multiple samples from the same cow over the lactation (Melzer et al., 2013). Hence, the aim of this study was to provide greater understanding of the changes in the metabolic profile throughout the whole lactation, involving several samples per cow and using untargeted global metabolomics with LC-MS/MS.

Coagulation, an important trait of milk technological quality, is influenced by lactation stage and other factors (Grandison et al., 1984; Auldist et al., 2002; Cassandro et al., 2008). Previously, Harzia et al. (2012) identified the difference in the metabolome of noncoagulating and coagulating milk, and differences in metabolic profiles of milk with different coagulation abilities have been reported by Sundekilde et al. (2011). The metabolites related to coagulation properties include citrate, choline, carnitine, lactose, and other oligosaccharides such as N-acetyllactosamine (NAcLac; Sundekilde et al., 2011; Harzia et al., 2012). Nevertheless, the change in milk metabolome throughout lactation and relationships with coagulation ability need to be investigated further; therefore, a second objective of this study was to identify correlations between metabolome and coagulation ability.

Received March 14, 2013.
Accepted June 19, 2013.
[1]Corresponding author: hedi.harzia@emu.ee

MATERIALS AND METHODS

Animal Management and Feeding

Animal use and care were in accordance with the Estonian Animal Protection Act. Cows in a loose housing system on the Estonian University of Life Sciences' experimental farm (Eerika Farm LLC, Märja, Estonia) were milked twice a day and fed TMR ad libitum year round. Three rations were used: ration 1 consisted of (DM) 40% silage, 58% concentrates, and 2% minerals, 11.3 MJ of ME, 160 g/kg CP, and 103 g/kg MP; ration 2 consisted (DM) 48% silage, 50% concentrates, and 2% minerals, 10.7 MJ of ME, 159 g/kg CP, and 97 g/kg MP; and ration 3 consisted of (DM) 73% silage, 25% concentrates, and 2% minerals, 9.7 MJ of ME, 143 g/kg CP, and 85 g/kg MP. The rations comprised grass (75%) and clover (25%) silage; barley, wheat, and maize meal; heat-treated rapeseed cake, limestone, sodium chloride, and a vitamin-mineral mix for lactating cows. After calving, the cows were fed with ration 2 up to 14 DIM, after which ration 1 was offered up to 6.5 mo of lactation or if milk production was still >30 kg/d. Thereafter, the cows were again fed ration 2 and, at 1 mo before drying off, ration 3 was applied.

Sample Collection and Analysis

Milk samples (40 mL; n = 306, 3.73 replicates per cow, 6, ..., 307 DIM) from 82 primi- and multiparous (n = 156 and 150, respectively) Estonian Holstein (n = 70), Estonian Red (n = 7), and Estonian Native (n = 5) dairy cows were collected from February 2011 to February 2012 once a month with in-line milk meters within the framework of regular animal recording.

Milk samples were analyzed for fat, protein, and SCC content at the laboratory of Estonian Animal Recording Centre. Curd firmness ($\mathbf{E_{30}}$, mm) and coagulation time (**RCT**, min) were analyzed with an Optigraph as described by Kübarsepp et al. (2005), and milk pH was recorded (pH meter, SevenMulti; Mettler Toledo GmbH, Greifensee, Switzerland). Milk samples were prepared and MS analyses were performed on an LC-MS/MS (3200 Q TRAP; AB Sciex Instruments, Framingham, MA) as described by Harzia et al. (2012).

Statistical Analysis

Mass spectral data were preprocessed by binning data to atomic mass unit resolution. The principal component analyses (**PCA**) were performed for m/z in positive and negative ion mode to discover the potential patterns in MS data for differently ionized metabolites. To study the alterations of milk metabolome during the lactation, the identified principal components (**PC**) were modeled following the general linear mixed (GLM) model:

$$y_{ijklm} = \mu + B_i + P_j + D_k + M_l + b_1 \times LDIM_{ijklm} + b_2 \times LDIM^2_{ijklm} + b_3 \times LDIM^3_{ijklm} + C_m + e_{ijklm},$$

where y_{ijklm} is the dependent variable, μ is the model intercept, B_i is the breed effect ($i = 1, 2, 3$), P_j is the parity effect ($j = 1, 2$; primi- and multiparous), D_k is the diet effect ($k = 1, 2, 3$), M_l is the milking time effect ($l = 1, 2$; morning and evening), $b_1 \times LDIM_{ijklm} + b_2 \times LDIM^2_{ijklm} + b_3 \times LDIM^3_{ijklm}$ is the third-order Lagrange polynomial of DIM, C_m is the random cow effect ($m = 1, \ldots, 82$), and e_{ijklm} is the model error. The same model was applied to study alterations in milk coagulation and production and composition traits during the lactation. To examine the relationships between different milk traits and milk metabolome during 3 different lactation stages—the beginning (the first 60 d), middle (mo 3 to 8), and the end of lactation (the last 60 d)—Spearman rank correlation analysis was performed considering also the binary dummy variables of parity and milking time.

Statistical significance between m/z intensities in the spectra of early and late lactation milk, morning and evening milkings, and the first and second to third parities was determined by Student's t-tests. Results were displayed on a volcano plot as the distribution of signal intensities relative to the mean at respective m/z values, which were ranked based on calculated statistical differences. Statistical analyses were performed with SAS software (version 9.1; SAS Institute Inc., Cary, NC) and with R 2.8.1 /BioConductor algorithms (R Development Core Team, 2009).

RESULTS AND DISCUSSION

Lactational Curves of Milk Characteristics

As the biological needs of the calf changes with age, milk composition alters as lactation progresses (Walstra, 1999). The GLM analyses of production, composition, and quality traits during lactation are presented in Figure 1; no abnormal dynamics were observed. Changes in milk yield and fat and protein contents were similar to those of Mucha and Strandberg (2011) and Stoop et al. (2009). Fat content changed contrarily to milk yield, declining during the first 100 d (from 4.49 to 3.85%) and

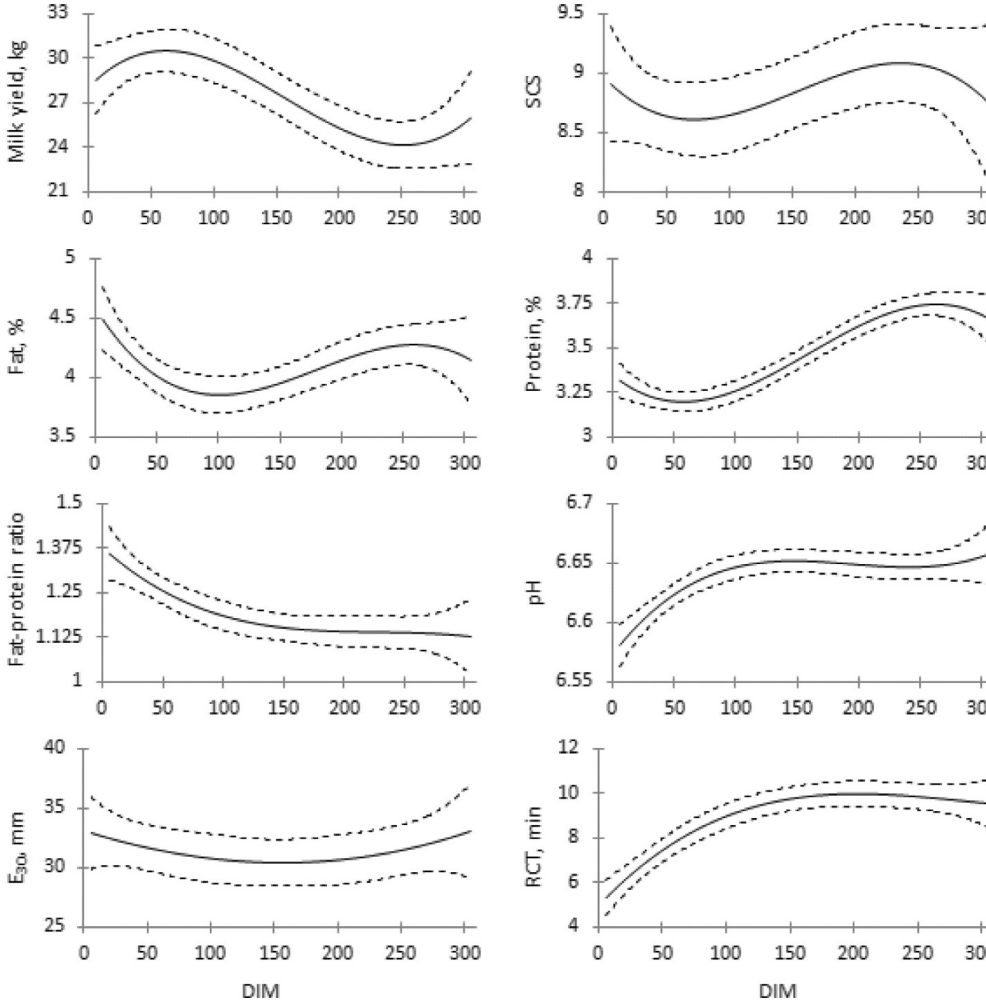


Figure 1. Change in milk production and composition traits (with 95% CI) during lactation estimated with third-order polynomial according to the linear mixed model considering additionally fixed effects of breed, parity (primi- and multiparous), diet, and milking time (morning and evening) and random effect of cow; SCS = [$\log_2$(SCC/100,000) + 3]. E_{30} = curd firmness; RCT = rennet coagulation time.

then increasing to d 259 (4.29%). Milk yield reached its peak at the end of mo 2 (30.5 kg/d) and then declined to d 251 (24.16 kg/d). Protein percentage declined over the first 57 d (from 3.32 to 3.20%) and then increased to peak level on d 263 (3.74%). Somatic cell score, calculated as SCS = [$\log_2$(SCC/100,000) + 3], had its lowest value at 76 DIM (SCS = 8.61) and highest value on 236 DIM (SCS = 9.08).

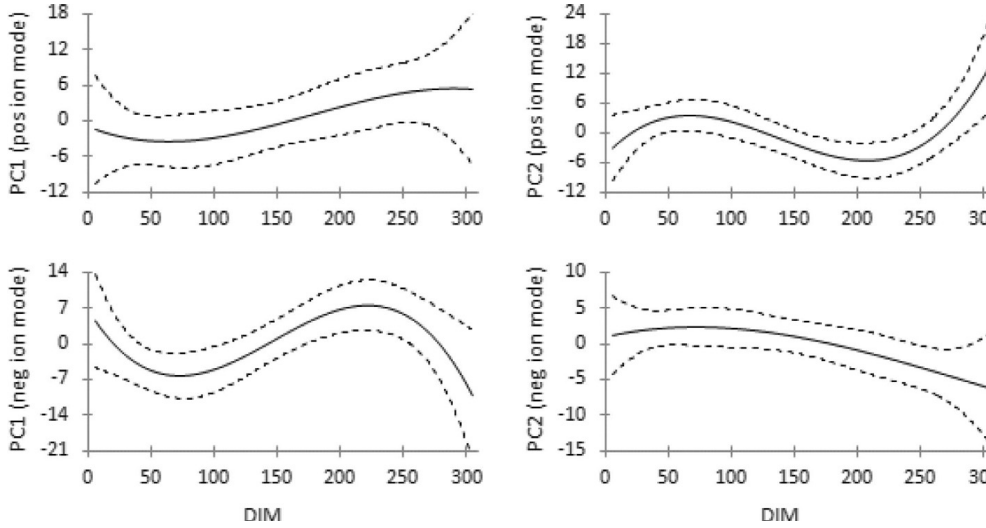


Figure 2. Results of principal component (PC) analyses of mass/charge ratio (m/z) in positive (pos) and negative (neg) ion mode. Change in the first 2 principal component values (with 95% CI) during lactation estimated with third-order polynomial according to the linear mixed model considering additionally fixed effects of breed, parity (primi- and multiparous), diet and milking time (morning and evening) and random effect of cow are presented.

Influence of Lactation on Metabolome

To improve our understanding of the change in metabolic profile throughout the whole lactation, PCA and GLM analyses were conducted. The results revealed 2 main patterns among MS signals from milk metabolites characterized by a linear or higher degree relationship to DIM (Figure 2, Table 1). One of the patterns reflected the difference between the first and second halves of lactation, whereas the other pattern (PC2 in positive and PC1 in negative ion mode) changed rapidly during the first 2 lactation months, achieved its initial value within the next 5 to 6 mo, and changed again, as at the beginning of the lactation, within mo 8 to 10 of lactation. Based on the loading plots, the first 2 PC accounted for more than 50% of the total variance of the measured signals either in positive or negative ion mode (Figure 3). The subsequent PC accounted for <8% of total variance each. As the first 2 PC were slightly related to almost every metabolic marker but at the same time no single metabolite existed with stronger contributions (Figure 3), the patterns described in Figure 2 are common to the whole milk metabolome and reflect general physiological changes during lactation. According to the modeling results of the PC (Table 1), no animal-specific component existed in the 2 first PC. This confirms that the discovered metabolic profiles are common to all cows; a common evolution for the direction of the metabolic profile from the first to the second month of lactation was observed previously (Ilves et al., 2012).

All modeled PC had different values at the beginning and at the end of the lactation. To obtain more information on these differences, the metabolic profile from the first 60 d was compared with that of the last 60 d of lactation. The results were visualized on volcano plots (Figure 4A and 4B) as the change in mean signal intensity versus the statistical significance of the change. The most significant ($P < 10^{-16}$; a >1.5-fold change in signal intensity) decrease during lactation was observed for a set of markers 225, 299, 397, 641, and 642 m/z (measured in positive mode), and 186, 241, 258–260, 289–290, 322, 357, 423–425, 466, 468, 486, 601–604, 622, 664, 699, 737, and 943–944 m/z (measured in negative mode). All these signals had a higher intensity in early lactation compared with late lactation. On the other hand, a significant increase of intensity during lactation showed only m/z 220 and 256 (Figure 4B). The findings of the current study expand previous results (Ilves et al., 2012) that indicated a decrease of several milk

Table 1. Modeling results of principal components of signals measured in positive and negative ion mode and milk coagulation, production, and composition traits

	Factor *P*-value[2]							Variance component	
Item[1]	Breed	Parity	Diet	Milking time	LDIM	LDIM^2	LDIM^3	Animal	Residual
$PC1_{PIM}$	0.50	**0.006**	0.99	**<0.001**	**0.032**	0.63	0.58	0.0	466.4
$PC2_{PIM}$	0.82	**<0.001**	0.49	**0.001**	0.81	**0.017**	**0.001**	5.1	238.6
$PC1_{NIM}$	0.75	0.41	0.38	**<0.001**	0.13	0.32	**0.003**	0.0	459.5
$PC2_{NIM}$	**<0.001**	0.86	0.06	**<0.001**	**0.006**	0.22	0.82	0.0	167.2
E_{30}, mm	**0.024**	0.27	0.88	**<0.001**	0.97	**0.049**	0.99	44.1	30.7
RCT, min	0.62	0.14	0.28	0.38	**<0.001**	**<0.001**	0.20	3.87	2.50
Fat:protein	**0.015**	0.16	**0.014**	**<0.001**	**<0.001**	**0.011**	0.43	0.0070	0.0276

[1]PC = principal component, PIM = positive ion mode, NIM = negative ion mode; E30 = curd firmness; RCT = milk (rennet) coagulation time.
[2]LDIM, LDIM^2, LDIM^3 = linear, quadratic, and cubic effects, respectively, of DIM modeled as Lagrange polynomial. Statistically significant effects ($P < 0.05$) are shown in bold type.

metabolites in negative mode by the start of mo 3 of lactation. Additionally, the decrease of signals 421–422, 426, 482, 606–608, 648, and 666 prevailing during the first 3 mo of lactation (Ilves et al., 2012) remained significant ($P < 0.001$, Figure 4B) throughout the lactation. Ilves et al. (2012) identified the signal with m/z 421 (negative mode) as lactose phosphate and noted that m/z 601–603 matched with m/z 421 spectra and could be a noncovalent complex with additional hexose or a covalent hexose + water complex.

Signal m/z 218 measured in negative mode (Figure 4B) was previously associated with feeding of crude glycerol and identified as pantothenic acid (Harzia et al., 2013). According to previous studies (Lardinois et al., 1944; Hollis et al., 1954; Hayes et al., 1967), the synthesis of pantothenic acid depends on the diet and increases as the supplementation of rapidly degradable carbohydrates is increased. In the present study, the higher intensity of pantothenic acid at the beginning of lactation could be related to the high level of concentrates (up to 62% of DM) in TMR during the high-yielding period.

The findings of the current study are consistent with those of Melzer et al. (2013), who found that day of lactation had a significant influence on 45.8% of the 190 metabolites studied; metabolite profiles from the beginning of lactation (21–50 DIM) clustered together, as did the later ones. However, only milk samples from

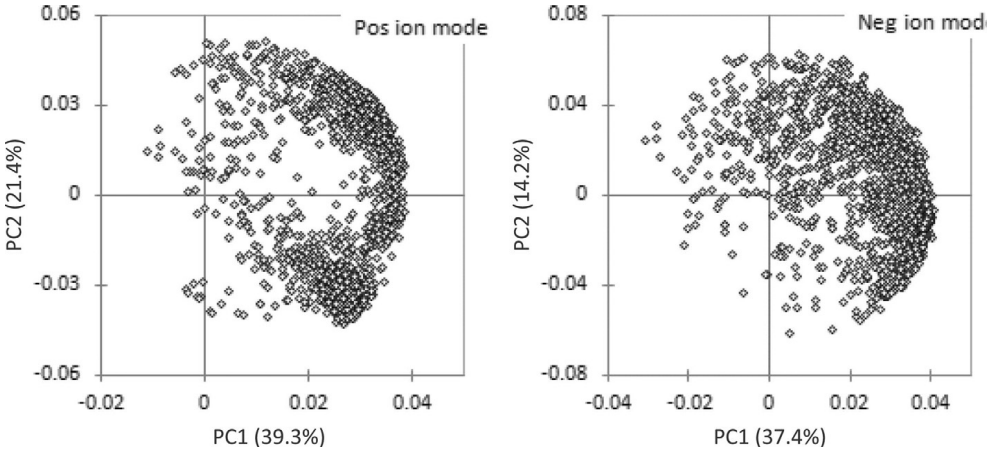


Figure 3. Loading plots of principal component (PC) analyses and their account of total variances of MS signals measured in positive (pos) and negative (neg) ion mode.

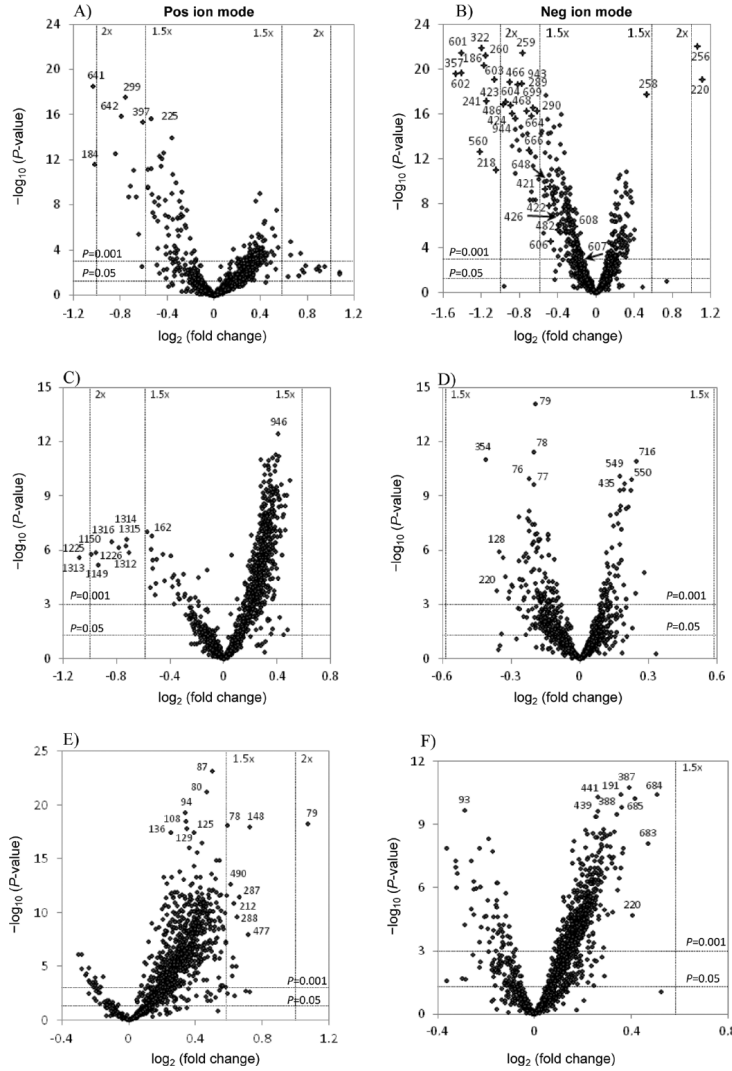


Figure 4. Volcano plots displaying the fold change in mass-to-charge ratio intensity (x-axis) versus the significance of the change (y-axis) for the (A, B) first and last 60 d of lactation in positive (pos; A) and negative ion mode (neg; B). Signals with the highest intensity and most significant change (decrease during lactation) in the first 60 d of lactation are in the upper left corner, signals with the highest and most significant intensity (increase during lactation) in the last 60 d of lactation are in the upper right corner; (C, D) as (A, B), but displaying difference of significance for primiparous and multiparous cows in positive (C) and negative (D) ion mode. Signals with the highest and most significant intensity in primiparous cows are in the upper left corner, signals with the highest and most significant intensity in multiparous cows are in the upper right corner; (E, F) as (A, B), but displaying difference of significance between morning and evening milk samples in positive (E) and negative (F) ion mode. The most significant signals with the highest intensity in the morning milk samples are in the upper left corner, the evening milk samples are in the upper right corner.

primiparous cows (1 sample per cow) during the period of 21 to 120 DIM were analyzed in Melzer et al. (2013).

The results of this study indicate that the milk metabolome differs between lactation periods. The physiological drive for changes could be the change in the needs of the suckling offspring, different milk yield during lactation, different metabolic state (i.e., utilization of fat and protein reserves for milk production at the beginning of lactation, the negative energy balance period), and, starting from conception, the metabolic needs of the fetus. In their review, Grummer and Rastani (2003) noted that, in 90% of cases, cows reach a positive energy balance by 63 d postpartum, the approximate time of the first turning point of the lactation curves of PC1 (negative mode) and PC2 (positive mode) in the present study. Hence, the results of our research implicitly support the idea that the milk metabolome is affected by the energy balance of the dairy cow. However, a limitation of our study is that the energy balance data for the cows are not available. Although milk fat to protein ratio (Figure 1), an indirect indicator used for energy balance estimation, was higher at the beginning of the lactation, it did not exceed the threshold level at 1.5 (Heuer et al., 2000) and was influenced by the ration of the cows (Table 1). Effect of pregnancy on milk yield and composition has been observed from the first day of gestation; the change, dependent on lactation stage (the influence being less noteworthy at the end of lactation; Olori et al., 1997), becomes significant from mo 5 of gestation onward (Olori et al., 1997; Roche, 2003). In the present study, the mean of the period open was 140 d and the change in the lactation curves of milk yield, fat and protein percentages, and PC1 (negative mode) and PC2 (positive mode) occurred around mo 3 of gestation.

Influence of Parity on Metabolome

As parity influenced both PC in positive ion mode (Table 1), the metabolic profiles of primi- and multiparous cows over the whole lactation were compared. The analyses in positive mode showed that signals with the highest and most significantly different intensities in primiparous cows compared with multiparous cows (Figure 4C) have m/z >1,000 Da. Markers with m/z of around 1,000 could indicate the presence of polymers, including peptides and lipids. This finding might therefore indicate the greater presence of peptides in the primiparous milk metabolome, which could be explained by the difference in milk protein content. In Estonian cows, depending on parity, protein content decreases with parity number (Jõudluskontrolli Keskus, 2012). The presence of the same set of signals did not depend on milking time or on beginning or end of lactation (Figure 4A, B, E, and F). In addition, signals with the highest and most significantly different intensities in multiparous cows compared with primiparous cows (Figure 4C and D) were detected (in positive mode 946 m/z, and in negative mode 435, 549, 550, and 716 m/z). The difference between a cow's metabolome in different parities could be related to differences in energy consumption and utilization in first-lactation dairy heifers compared with older cows. First-lactation dairy cows are still growing and they do not give as much milk as multiparous cows.

Influence of Milking Time on Metabolome

As expected (Gilbert et al., 1973; Palmer et al., 1994), milking time had an effect on almost all variables studied (Table 1). The PC values were significantly influenced by milking time; the estimated values of the first PC in both positive and negative ion modes were higher at evening milking and those of the second PC were higher at morning milking, according to the GLM. All these effects remained the same after omitting the nonsignificant factors. The metabolic profiles of morning and evening milk samples were compared and a significant difference was observed in positive ion mode (Figure 4E), with signal 79 m/z having the highest and significantly different intensity in evening milk. In negative ion mode, m/z 93 had the highest intensity in morning milking. A significant increase for signal 191 m/z, indicating citrate, measured in negative ion mode, was observed in evening milk (Figure 4F). According to Faulkner and Peaker (1982), citrate concentration is positively associated with the fat content of milk, which is in good accord with our results (data not shown).

Metabolome Influence on Milk Coagulation

The GLM results of coagulation parameters are presented in Figure 1 and Table 1. The estimated curve for E_{30} was persistent, without noticeable peaks, and maintained favorable values throughout the lactation. In agreement with the results of Ostersen et al. (1997), the estimated E_{30} was optimal (>32 mm) at the beginning and end of lactation and lowest during mid lactation (30.42 mm on d 157). Greater variation in E_{30} was observed by Vallas et al. (2010), but trajectories describing RCT were consistent in the 2 studies. Estimated RCT increased to the end of mid lactation, being lowest during the first 18 d (<6 min) and highest during mid lactation (d 172–238, mean value between 9.90 and 9.99 min). At the end of lactation, RCT decreased until it reached 9.55 min. Increase in RCT has previously (Grandison et al., 1984; Ostersen et al., 1997; Kübarsepp et al., 2005) been associated with in-

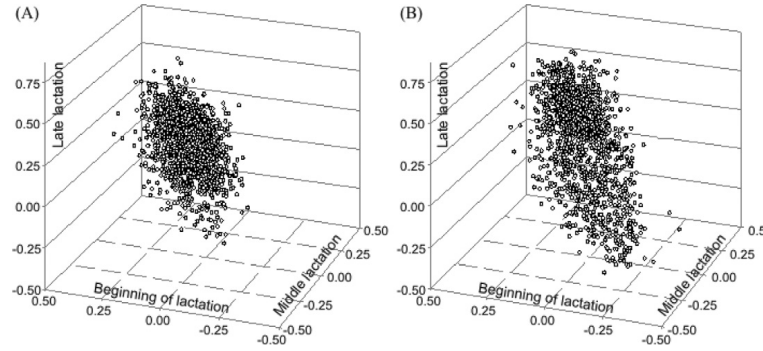


Figure 5. Spearman rank correlation coefficients between curd firmness (E_{30}) and signals measured in (A) positive and (B) negative ion mode; each dot corresponds to 1 signal and represents its correlations with E_{30} at 3 different lactation stages.

creased pH. Lactational changes in curd firmness have previously been associated with variation in milk composition, mainly with milk protein and their fraction contents (Guinee, 2003; Jõudu et al., 2007, 2008). In addition to general lactational dynamics, the modeling results of milk coagulation traits (Table 1) revealed that the values of these traits are strongly animal specific: the proportion of variance related to individuals varied from 0.3 to 0.6. In addition, E_{30} values were influenced by milking time: firmer curd and shorter RCT were observed at evening milking.

To verify the second objective, we studied the correlations between milk coagulation properties and milk metabolome at different lactation stages. Although no strong relationships were found, the direction and strength of the relationships were more or less the same at the beginning, middle, and end of the lactation (Figure 5). Two biomarkers were found in positive ion mode (m/z 197 and 342) and 8 in negative ion mode (m/z = 612, 737, 835, 836, 902, 1000, 1038, and 1079), which had at least intermediate positive relationships (correlation coefficient r >0.3) with E_{30} at every lactation stage. Both positive signals and 4 negative (902, 1000, 1038, and 1079) signals were positively correlated (correlation coefficient r >0.3) with protein throughout the lactation. We found no signals that had at least intermediate relationships with RCT at lactation stage.

CONCLUSIONS

The results revealed 2 main patterns among MS signals from milk metabolites characterized by a linear or a higher degree relationship to DIM. Sets of specific markers for lactation periods, parity, and milking time were ascertained. In addition, correlations between certain metabolites and coagulation ability persisted throughout lactation; no markers were linked to RCT.

ACKNOWLEDGMENTS

This work was supported by the targeted finance project SF0170165s08, Estonian Science Foundation grant 7856, and by the Bio-Competence Centre of Healthy Dairy Products co-financed by the European Community's Regional Development Fund within the framework of the Competence Centre Program of the Enterprise Estonia, project no. EU30002.

REFERENCES

Auldist, M., C. Mullins, B. O'Brien, B. T. O'Kennedy, and T. Guinee. 2002. Effect of cow breed on milk coagulation properties. Milchwissenschaft 57:140–143.

Cassandro, M., A. Comin, M. Ojala, R. Dal Zotto, M. De Marchi, L. Gallo, P. Carnier, and G. Bittante. 2008. Genetic parameters of milk coagulation properties and their relationships with milk yield and quality traits in Italian Holstein cows. J. Dairy Sci. 91:371–376.

Faulkner, A., and M. Peaker. 1982. Reviews of the progress of dairy science: Secretion of citrate into milk. J. Dairy Res. 49:159–169.

Gilbert, G. R., G. L. Hargrove, and M. Kroger. 1973. Diurnal variations in milk yield, fat yield, milk fat percentage, and milk protein percentage of Holstein-Friesian cows. J. Dairy Sci. 56:409–410.

Grandison, A. S., G. D. Ford, A. J. Owen, and D. Millard. 1984. Chemical composition and coagulating properties of renneted Friesian milk during the transition from winter rations to spring grazing. J. Dairy Res. 51:69–78.

Grummer, R. R., and R. R. Rastani. 2003. Review: When should lactating dairy cows reach positive energy balance? Prof. Anim. Sci. 19:197–203.

Guinee, T. P. 2003. Role of protein in cheese and cheese products. Pages 1083–1174 in Advanced Dairy Chemistry 1: Proteins, Vol. 1. Part B. P. F. Fox and P. L. H. McSweeney, ed. Kluver Academic/ Plenum Publisher, New York, NY.

Harzia, H., K. Kilk, T. Ariko, M. Kass, U. Soomets, I. Jõudu, T. Kaart, D. Arney, O. Kärt, and M. Ots. 2013. Crude glycerol as

glycogenic precursor in feed: Effects on milk coagulation properties and metabolic profiles of dairy cows. J. Dairy Res. 80:190–196.
Harzia, H., K. Kilk, I. Jõudu, M. Henno, O. Kärt, and U. Soomets. 2012. Comparison of the metabolic profiles of noncoagulating and coagulating bovine milk. J. Dairy Sci. 95:533–540.
Hayes, B. W., G. E. Mitchell Jr., and C. O. Little. 1967. Factors affecting concentrations of B-vitamins in ruminal fluid in vitro. Int. Z. Vitaminforsch. 37:348–353.
Heuer, C., W. M. Van Straalen, Y. H. Schukken, A. Dirkzwager, and J. P. T. M. Noordhuizen. 2000. Prediction of energy balance in a high-yielding dairy herd in early lactation: Model development and precision. Livest. Prod. Sci. 65:91–105.
Hollis, L., C. F. Chappel, R. MacVicar, and C. K. Whitehair. 1954. Effect of ration on vitamin synthesis in rumen of sheep. J. Anim. Sci. 13:732–738.
Ilves, A., H. Harzia, K. Ling, M. Ots, U. Soomets, and K. Kilk. 2012. Alterations in milk and blood metabolomes during the first months of lactation in dairy cows. J. Dairy Sci. 95:5788–5797.
Jõudluskontrolli Keskus. 2012. Jõudluskontrolli aastaaruanded 2011 [Animal Control Annual Reports for 2011]. Elmatar, Tartu, Estonia.
Jõudu, I., M. Henno, T. Kaart, T. Püssa, and O. Kärt. 2008. The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows. Int. Dairy J. 18:964–967.
Jõudu, I., M. Henno, S. Värv, T. Kaart, O. Kärt, and K. Kalamees. 2007. Milk protein genotypes and milk coagulation properties of Estonian native cattle. Agric. Food Sci. 16:222–231.
Klein, M. S., M. F. Almstetter, G. Schlamberger, N. Nürnberger, K. Dettmer, P. J. Oefner, H. H. D. Meyer, S. Wiedemann, and W. Gronwald. 2010. Nuclear magnetic resonance and mass spectrometry-based milk metabolomics in dairy cows during early and late lactation. J. Dairy Sci. 93:1539–1550.
Klein, M. S., N. Buttchereit, S. P. Miemczyk, A. K. Immervoll, C. Louis, S. Wiedemann, W. Junge, G. Thaller, P. J. Oefner, and W. Gronwald. 2012. NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. J. Proteome Res. 11:1373–1381.
Kübarsepp, I., M. Henno, O. Kärt, and T. Tupasela. 2005. A comparison of the methods for determination of the rennet coagulation properties of milk. Acta Agric. Scand. A Anim. Sci. 55:145–148.
Lardinois, C. C., R. C. Mills, C. A. Elvehjem, and E. B. Hart. 1944. Rumen synthesis of the vitamin B complex as influenced by ration composition. J. Dairy Sci. 27:579–583.
Malossini, F., S. Bovolenta, C. Piras, M. DallaRosa, and W. Ventura. 1996. Effect of diet and breed on milk composition and rennet coagulation properties. Ann. Zootech. 45:29–40.
Melzer, N., D. Wittenburg, S. Hartwig, S. Jakubowski, U. Kesting, L. Willmitzer, J. Lisec, N. Reinsch, and D. Repsilber. 2013. Investigating associations between milk metabolite profiles and milk traits of Holstein cows. J. Dairy Sci. 96:1521–1534.
Mucha, S., and E. Strandberg. 2011. Genetic analysis of milk urea nitrogen and relationships with yield and fertility across lactation. J. Dairy Sci. 94:5665–5672.
Olori, V. E., S. Brotherstone, W. G. Hill, and B. J. McGuirk. 1997. Effect of gestation stage on milk yield and composition in Holstein Friesian dairy cattle. Livest. Prod. Sci. 52:167–176.
Ostersen, S., J. Foldager, and J. E. Hermansen. 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. J. Dairy Res. 64:207–219.
Palmer, R. W., E. L. Jensen, and A. R. Hardie. 1994. Removal of within-cow differences between morning and evening milk yields. J. Dairy Sci. 77:2663–2670.
R Development Core Team. 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Accessed Sep. 15, 2012. http://www.R-project.org.
Roche, J. R. 2003. Effect of pregnancy on milk production and bodyweight from identical twin study. J. Dairy Sci. 86:777–783.
SAS Institute. 2008. SAS/STAT 9.1 User's Guide. SAS Inst. Inc., Cary, NC.
Stoop, W. M., H. Bovenhuis, J. M. L. Heck, and J. A. M. van Arendonk. 2009. Effect of lactation stage and energy status on milk fat composition of Holstein-Friesian cows. J. Dairy Sci. 92:1469–1478.
Sundekilde, U. K., P. D. Frederiksen, M. R. Clausen, L. B. Larsen, and H. C. Bertram. 2011. Relationship between the metabolite profile and technological properties of bovine milk from two dairy breeds elucidated by NMR-based metabolomics. J. Agric. Food Chem. 59:7360–7367.
Vallas, M., H. Bovenhuis, T. Kaart, K. Parna, H. Kiiman, and E. Parna. 2010. Genetic parameters for milk coagulation properties in Estonian Holstein cows. J. Dairy Sci. 93:3789–3796.
Walstra, P. 1999. Dairy Technology: Principles of Milk Properties and Processes. Food Science and Technology No. 90. Marcel Dekker, New York, NY.

CURRICULUM VITAE

First name	Hedi
Surname	Harzia
Date of birth	December 23, 1979
Institution	Estonian University of Life Sciences Institute of Veterinary Medicine and Animal Sciences, Department of Animal Nutrition and Products Quality, Laboratory of Milk Quality Kreutzwaldi 48, Tartu 51006, Estonia
Position	Researcher

Education

2008 – 2013	Estonian University of Life Sciences Institute of Veterinary Medicine and Animal Sciences, PhD studies
2005 – 2007	University of Tartu, Faculty of Physics and Chemistry, Environmental Technology, *MSc*
2001 – 2005	University of Tartu, Faculty of Physics and Chemistry, Environmental Technology, *BSc*
1998 – 2000	University of Tartu Faculty of Medicine, medicine
1986 – 1998	Loo Secondary School

Work experience

2010 – 2013	Estonian University of Life Sciences, researcher
2008 – 2013	Bio-Competence Centre of Healthy Dairy Products LLC, researcher
2007 – 2010	Estonian University of Life Sciences, extraordinary researcher
2006 – 2007	A. Le Coq, technician-microbiologist

Participation in research projects

2009 – 2013	Sustainable nutritional strategies to produce compositionally designed milk to promote human health (8-2/T9084VLVL)
2008 – 2012	Cattle health and welfare - aspects of precision livestock farming (SF0170165s08)

2008 – 2010	Developing and implementing of the prevention system for lipid-related metabolic disorders, based on milk metabolites (8-2/T8009VLVL)
2008 – 2009	Sustainable nutritional strategies to produce compositionally designed milk to promote human health (8-2/T8134VLVL)
2004 – 2007	The reasons and indicator parameters of phytotoxicity of oil shale semi-coke and plant growth substrates produced from it (ETF5811)

CURRICULUM VITAE

Eesnimi	Hedi
Perekonnanimi	Harzia
Sünniaeg	23. detsember 1979
Töökoht	Eesti Maaülikool veterinaarmeditsiini ja loomakasvatuse instituut, söötmise osakond, piima kvaliteedi uurimise labor Kreutzwaldi 48, Tartu 51006, Estonia
Ametikoht	Teadur

Haridus

2008 – 2013	Eesti Maaülikool veterinaarmeditsiini ja loomakasvatuse instituut, doktorantuur
2005 – 2007	Tartu Ülikool füüsika-keemiateaduskond, keskkonnatehnoloogia, *MSc*
2001 – 2005	Tartu Ülikool füüsika-keemiateaduskond, keskkonnatehnoloogia, *BSc*
1998 – 2000	Tartu Ülikool arstiteaduskond, arstiteadus
1986 – 1998	Loo Keskkool

Teenistuskäik

2010 – 2013	Eesti Maaülikool, teadur
2008 – 2013	Tervisliku Piima Biotehnoloogiate Arenduskeskus OÜ, teadur
2007 – 2010	Eesti Maaülikool, erakorraline teadur
2006 – 2007	AS A. Le Coq, laborant-mikrobioloog

Osalemine uurimisprojektides

2009 – 2013	Jätkusuutlikud söötmise strateegiad disainitud koostisega tervisliku piima tootmiseks (8-2/T9084VLVL)
2008 – 2012	Veiste tervise ja heaolu uurimine täppispidamise aspektist (SF0170165s08)
2008 – 2010	Piima metaboliitidel põhineva rasva ainevahetushaiguste ennetamise süsteemi väljatöötamine ja juurutamine Eestis jõudluskontrolli süsteemis (8-2/T8009VLVL)

2008 – 2009	Jätkusuutlikud söötmise strateegiad disainitud koostisega tervisliku piima tootmiseks (8-2/T8134VLVL)
2004 – 2007	Põlevkivi poolkoksi ja sellest valmistatavate taimekasvusubstraatide fütotoksilisuse põhjused ja indikaatorparameetrid (ETF5811)

LIST OF PUBLICATIONS

1.1. Publications indexed in the ISI Web of Science database

Harzia, H., Ilves, A., Ots, M., Henno, M., Jõudu, I., Kaart, T., Ling, K., Kärt, O., Kilk, K., Soomets, U. 2013. Alterations in milk metabolome and coagulation ability during the lactation of dairy cows. *Journal of Dairy Science,* 96 (10), 6440-6448.

Harzia, H., Kilk, K., Ariko, T., Kass, M., Soomets, U., Jõudu, I., Kaart, T., Arney, D., Kärt, O., Ots, M. 2013. Crude glycerol as glycogenic precursor in feed; effects on milk coagulation properties and metabolic profiles of dairy cows. *Journal of Dairy Research*, 80 (2), 190 – 196.

Harzia, H., Kilk, K., Jõudu, I., Henno, M., Kärt, O., Soomets, U. 2012. Comparison of the metabolic profiles of noncoagulating and coagulating bovine milk. *Journal of Dairy Science*, 95(2), 533 – 544.

Ilves, A., Harzia, H., Ling, K., Ots, M., Soomets, U., Kilk, K. 2012. Alterations in milk and blood metabolomes during the first months of lactation in dairy cows. *Journal of Dairy Science*, 95(10), 5788 – 5797.

Harzia, H., Orupõld, K., Habicht, H., Tenno, T. 2007. Leaching behaviour of oil shale semicoke: sulphur species. *Oil Shale*, 24(4), 583 – 589.

3.4. Papers published in the proceedings of international conferences

Ilves, A., Harzia, H., Ling, K., Kärt, O., Soomets, U., Kilk, K. 2011. Negative energy balance induced changes in milk metabolome of dairy cows. In: International Oskar Kellner Symposium. Metabolic Flexibility in Animal and Human Nutrition. (Eds. C. C. Metges, H. M. Hammon, B. Kuhla, M. Röntgen, W. B. Souffrant), 9. – 11.09.2011, Alstadt-Druck GmbH, Germany, 55 – 55.

5.2. Thesis published in the proceedings of international conferences

Harzia, H., Kilk, K., Jõudu, I., Henno, M., Kärt, O., Soomets, U. 2012. Metabolic profiles of non- and coagulating bovine milk. In: NJF 439th seminar, Dairy production in modern loose housing cowsheds - practical implications and future challenges. (Ed. A. Kaasik), 2. – 4.05.2012, Tartu, Estonia, 44 – 45.

Harzia, H., Kilk, K., Jõudu, I., Henno, M., Kärt, O., Soomets, U. 2012. Metabolome of non- and coagulating cows milk. In: Book of abstracts ESR workshop "Feed your knowledge!" (Eds. A. Tres, T. Altintzoglou, K. Livingstone, T. Frankic, L. Pinotti), 7. – 8.06.2012, Barcelona, Spain, 44.

Jõudu, I., Henno, M., Ots, M., Harzia, H., Värv, S., Vallas, M. 2012. Review of studies concerning milk rennet coagualtion properties in Estonia during last decade. – 7th Baltic Conference on Food Science and Technology, Foodbalt-2012, 17-18.05.2012, Kaunas Technology University, Kaunas, Lithuania, 77.

Jõudu, I., Jaakson, H., Henno, M., Harzia, H., Ots, M. 2012. Effect of dietary cation-anion difference on milk rennet coagulation properties. In: International Scientific Conference "Physiology of Livestock" Programme and Abstracts: International Scientific Conference "Physiology of Livestock", 27-28.09.2012, Lithuanian University of Health Science Veterinary Academy, Kaunas, Lithuania, 29 - 30.

6.3. Popular science articles

Ots, M., Ariko, T., Harzia, H., Samarütel, J., Henno, M., Kärt, O. 2010. Mis võimalusi pakub lähitulevik lehmade tervise ja söötmise hindamisel. Piimafoorum, Paide, Eesti, 35 - 37 (In Estonian)

VIIS VIIMAST KAITSMIST

LIINA ARUS

THE INFLUENCE OF CULTIVAR AND NATURAL ENEMIES ON RASPBERRY BEETLE (*Byturus tomentosus* De Geer)
SORDI JA LOODUSLIKE VAENLASTE MÕJU VAARIKAMARDIKALE (*Byturus tomentosus* De Geer)
Prof. *emer.* **Anne Luik**
4. oktoober 2013

ARNE KÜÜT

CHARACTERISTICS OF BIOETHANOL FUEL OBATAINED FROM LIGNOCELLULOSE BIOMASS IN INTERNAL COMBUSTION RECIPROCATING ENGINES WITH SPARK- AND COMPRESSIONIGNITION
LIGNOTSELLULOOSSEST BIOMASSIST SAADAVATE BIOETANOOLKÜTUSTE KARAKTERISTIKUD SÄDE- JA SURVESÜÜTEGA SISEPÕLEMISMOOTORITES
Professor **Jüri Olt**
8. november 2013

LEILA MAINLA

CHANGES IN THE BIOCHEMICAL COMPOSITION OF APPLE (*Malus domestica* Borkh.) FRUITS DEPENDING ON ROOTSTOCK AND CALCIUM TREATMENT
MUUTUSED ÕUNTE BIOKEEMILISES KOOSTISES SÕLTUVALT AED-ÕUNAPUU (*Malus domestica* Borkh.) POOKEALUSEST JA KALTSIUMIGA VÄETAMISEST
Professor **Kadri Karp**, dotsent **Ulvi Moor**
15. november 2013

FLOORTJE VODDE

MICROSITES AND TREE REGENERATION DYNAMICS: PROLONGED STORM EFFECTS IN HEMIBOREAL MIXED FOREST
TORMIKAHJUSTUSTE KÄIGUS TEKKINUD MIKROALADE DÜNAAMIKA JA HÄIRINGUJÄRGNE PUURINDE UUENEMINE HEMIBOREAALSES SEGAMETSAS
Professor **Kalev Jõgiste**, professor **Frits Mohren** (*Wageningen University, The Netherlands*)
16. detsember 2013

MEELIS SEEDRE

DISTURBANCE EFFECTS ON BOREAL FOREST ECOSYSTEM CARBON DYNAMICS
HÄIRINGUREŽIIMI MÕJU BOREAALSE METSAÖKOSÜSTEEMI SÜSINIKUVOOGUDELE
Professor **Kalev Jõgiste**, prof. **Han Chen** (*Lakehead University, Canada*)
16. detsember 2013

ISBN 978-9949-536-07-8 (trükis)
ISBN 978-9949-536-08-5 (pdf)

roheline trükis

Trükitud taastoodetud paberile looduslike trükivärvidega © Ecoprint